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Cardiomyocyte-specific overexpression of human stem cell factor protects against myocardial ischemia and reperfusion injury☆

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ABSTRACT

Background: Cardiomyocyte-specific overexpression of human membrane-associated stem cell factor (hSCF) improves cardiac function post-myocardial infarction. However, whether hSCF overexpression protects the heart from ischemia and reperfusion (I/R) injury is unknown. We aimed to investigate the effects of cardiomyocyte-specific overexpression of hSCF on cardiac injury after acute myocardial I/R and related cellular and molecular signaling mechanisms.

Methods and results: Wild-type (WT) and hSCF/tetracycline transactivator (tTA) transgenic mice (hSCF/tTA) were subjected to myocardial ischemia for 45 min followed by 3 h of reperfusion. Infarct size and myocardial apoptosis were decreased in hSCF/tTA compared to WT mice ($P < 0.05$). Furthermore, these cardioprotective effects in the hSCF/tTA mice were abrogated by doxycycline, which turned off hSCF overexpression, and by a PI3 kinase inhibitor LY294002. Myocardial expression of insulin-like growth factor (IGF)-1 and hepatocyte growth factor (HGF), which are upstream activators of Akt signaling, was significantly increased in hSCF/tTA compared to WT mice after I/R ($P < 0.05$), and was associated with higher number of *c-kit*$^+$ cardiac stem cells (CSCs) ($P < 0.05$). Inhibition of *c-kit* signaling by ACK2 treatment abolished these protective effects in hSCF/tTA mice.

Conclusions: Cardiomyocyte-specific overexpression of hSCF protects the heart from I/R injury. The cardioprotective effects of hSCF overexpression are mediated by increased *c-kit*$^+$ CSCs, enhanced growth factor expression and activation of Akt signaling pathway.

1. Introduction

Ischemic heart disease is the leading cause of death worldwide [1]. Following acute myocardial infarction (MI), re-establishing coronary blood flow by thrombolysis or angioplasty is required to rescue the ischemic myocardium. However, reperfusion can also induce myocyte death by activation of deleterious signaling cascades [2]. Therapeutic strategies on cardiac protection from ischemia and reperfusion (I/R) injury are urgently demanded. Studies on pre- and post-conditioning have shown that I/R injury is able to initiate several pro-survival kinase cascades including phosphatidylinositide 3-kinase (PI3K)/Akt signaling pathway [3]. Constitutively active Akt mutant (myr-Akt) has been shown to decrease myocardial apoptosis, infarct size and preserve cardiac function in rats after I/R [4]. Akt activation leads to phosphorylation of multiple protein targets including endothelial nitric oxide synthase (eNOS) [5], which promotes cell survival [6]. Additionally, the beneficial effects of growth factors including vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF-1) and hepatocyte growth factor (HGF) [7], and pharmacological treatments such as statins and erythropoietin [5,8] on ischemic injury are mainly a result of Akt activation.

Stem cell factor (SCF) is a glycosylated trans-membrane protein widely expressed by many cells and tissues including stromal cells, fibroblasts, endothelium, and myocardium [9,10]. It plays a critical role in the survival, proliferation, mobilization, and adhesion of all *c-kit* expressing cells including hematopoietic stem cells, endothelial progenitor cells (EPCs), and cardiac stem cells (CSCs) [11–13]. Alternative splicing of SCF leads to two isoforms: soluble and membrane-associated isoforms. Membrane-associated SCF, the dominant isoform *in vivo*, is able to induce more persistent receptor activation and is more effective in promoting long-term support of target cell survival [14,15]. Binding of *c-kit* with SCF through autocrine or paracrine actions leads to oligomerization and auto-phosphorylation of the receptor which activates multiple downstream signaling pathways including the PI3K/Akt pathway. PI3K/Akt

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pathway activated by SCF is responsible for *c-kit*$^+$ cell proliferation, differentiation, adhesion, secretion, survival, and actin cytoskeletal reorganization [11,16,17].

We have recently demonstrated that cardiomyocyte-specific overexpression of human stem cell factor (hSCF) improves cardiac function and animal survival post-MI in mice [18]. The beneficial effects are due to increased *c-kit*$^+$ cell recruitment, enhanced growth factors expression, decreased myocardial apoptosis, increased angiogenesis and impaired cardiac remodeling [18]. However, whether hSCF overexpression protects the heart from acute I/R injury remains to be determined. This is an important question as limiting myocardial ischemic injury is critical to preserve functional myocardium and prevent subsequent progression to heart failure. In the present study, we hypothesized that inducible cardiomyocyte-specific overexpression of hSCF protects the heart from I/R injury. To test this hypothesis, a transgenic mouse that overexpresses hSCF in cardiomyocytes under the control of a Tet-off system was employed [19]. We demonstrated that cardiomyocyte-specific hSCF overexpression protects the heart from I/R injury via increased *c-kit*$^+$ cell activation, enhanced growth factor expression and Akt activation.

2. Methods

2.1. Animals

The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology. Animals in this study

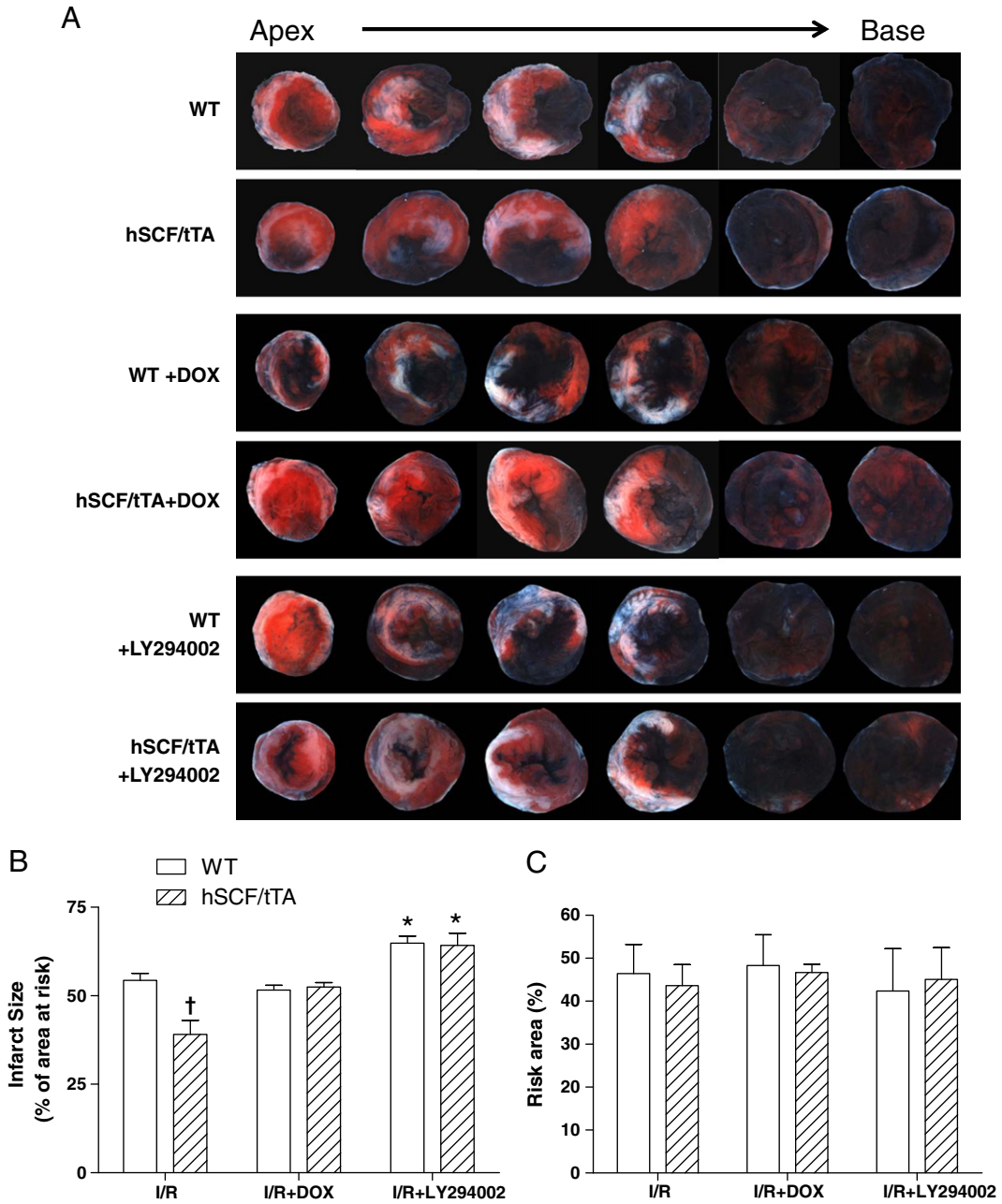

Fig. 1. Effects of hSCF overexpression on infarct size after myocardial I/R. Overexpression of hSCF significantly improved infarct size in hSCF/tTA compared to WT mice. Turning off the hSCF expression by DOX treatment or inhibition of PI3 kinase by LY294002 abrogated the reduction of infarct size in hSCF/tTA mice. A: Representative TTC stained heart sections (apex to base) after I/R from each corresponding group. Infarct area is shown white. B: Infarct size expressed as percent of the weight of the infarct to the area at risk. C: Area at risk after I/R. Data are mean ± SEM, $n = 5$–7 per group. Two-way ANOVA followed by Bonferroni test: $^*P < 0.05$ vs. I/R; † $P < 0.05$ vs. WT.

were handled in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). The conditional cardiac-specific membrane-associated human SCF overexpressing hSCF/tTA transgenic mice were generated using α-myosin heavy chain promoter as described previously [18,19]. The first generation of hSCF/tTA mice was from C57BL6/CBA background and backcrossed to C57BL/6 for 16 generations when the present study started. In total, 87 adult male hSCF/tTA and 95 WT mice (3–4 months old) were used.

2.2. Myocardial I/R

Induction of myocardial I/R was performed as previously described [20]. Mice were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (12.5 mg/kg) mixture and then intubated and artificially ventilated with a respirator (SAR-830, CWE, Ardmore, PA, USA). The heart was exposed by a left intercostal thoracotomy. The pericardium was opened, and the left coronary artery was occluded for 45 min by positioning a suture (8-0) around it with a PE-10 tubing. The tubing was then removed and the suture was loosened to allow reperfusion for 3 h. The lungs were hyperinflated using positive end-expiratory pressures (3 cm H_2O), and the thorax was closed. To inhibit Akt signaling, animals were treated with a PI3 kinase inhibitor LY294002 (Sigma, 7.5 mg/kg body weight, IP) 15 min before reperfusion. To inhibit *c-kit* signaling, mice were treated with a neutralizing antibody ACK2 (100 μg, IP, eBioscience, San Diego, CA, USA) 15 min before ischemia [21]. To inhibit HGF receptor (c-Met) signaling, mice were treated with a c-Met inhibitor crizotinib (15 mg/kg body weight, IP, Selleckchem, Houston, TX, USA) 15 min before ischemia [22]. To turn off hSCF overexpression, the hSCF/tTA mice were treated daily with doxycycline (DOX, 0.2 mg/ml in drinking water) starting 2 weeks before surgeries [18].

2.3. Measurement of infarct size

Infarct size was calculated by infarct area to ischemic area ratio normalized by tissue weight [20]. Briefly, the coronary artery was re-ligated after 3 h reperfusion. Evans blue dye solution (1% in PBS) was perfused through coronary artery via the cannulated aorta to distinguish ischemic and non-ischemic areas of the heart. Hearts were cut into four transverse slices from the apex to base and stained with 1.5% triphenyltetrazolium chloride (TTC, Sigma, St. Louis, MO, USA) for 30 min at room temperature. Sections were weighed and photographed. The non-ischemic area, area at risk, and infarct area were measured using SigmaScan Pro software.

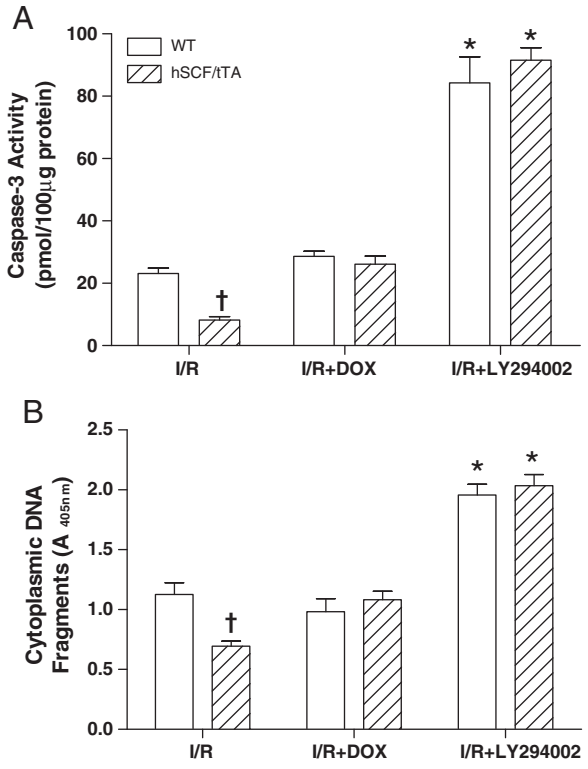

Fig. 2. Myocardial apoptosis in peri-infarct area after I/R. Overexpression of hSCF in hSCF/tTA mice showed significantly less apoptosis as measured by caspase-3 activity (A) and cell death ELISA (B). These effects were abrogated by turning off the expression of hSCF by DOX treatment or blocking PI3K by LY294002 treatment in hSCF/tTA mice. Data are mean ± SEM, $n = 5$. Two-way ANOVA followed by Bonferroni test: $^*P < 0.05$ vs. I/R; † $P < 0.05$ vs. WT.

2.4. Determination of myocardial apoptosis

Caspase-3 activity and cytoplasmic histone-associated DNA fragments were measured using caspase-3 cellular activity assay kit (BIOMOL, Plymouth Meeting, PA) and cell death detection ELISA (Roche, Mississauga, ON), respectively as we previously described [18]. Briefly, heart tissues from the ischemic area were removed and homogenized. After 5-min centrifuge at 12,000 ×g at 4 °C, supernatants were collected and protein concentrations were measured. To assess caspase-3 activity, supernatant from each heart containing 200 μg proteins was loaded into 96-well plate and incubated at 37 °C for 16 h in the presence of the caspase-3 substrate Ac-DEVD-AMC (20 μM) or Ac-DEVD-AMC plus the inhibitor Ac-DEVD-CHO (4 μM). Fluorescent intensity (excitation at 360 nm and emission at 460 nm) was measured using a Spectra-Max M5 micro-plate reader (Molecular Devices, Sunnyvale, CA, USA). Data were expressed as amounts of AMC substrate cleaved per 100 μg of protein. To assess cytoplasmic histone-associated DNA fragments, supernatant containing 20 μg proteins was incubated with anti-histone antibody coated 96-well plate for 90 min. The sample solution was removed and horseradish peroxidase-conjugated anti-DNA antibody was added and incubated for 90 min. The color was developed by adding peroxidase substrate (ABTS) and detected by a Spectra-Max micro-plate reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nM wavelength.

2.5. Western blot analysis

Myocardial HGF and IGF-1 expression, and phosphorylated/total Akt and eNOS in the peri-infarct area of the myocardium were measured by western blot analysis. Briefly, 40–60 μg of protein was separated by 10%–15% SDS-PAGE gel and transferred to nitrocellulose membranes, and blots were probed with antibodies against Akt (1:1000, ABM, Vancouver, BC, Canada), phosphorylated Akt (Ser473, 1:1000, ABM, Vancouver, BC, Canada), eNOS (1:500, Cell Signaling, Danvers, MA, USA), phosphorylated eNOS (1:500, Cell Signaling, Danvers, MA, USA), α-actinin (1:2500, Sigma, Oakville, ON, Canada), HGF (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), IGF-1 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or GAPDH (1:3000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were then washed and probed with horseradish peroxidase conjugated secondary antibodies (1:3000, Bio-Rad, Hercules, CA, USA), and detected by using an ECL detection method.

2.6. Growth factor mRNA expression

Total mRNA was isolated from LV heart tissue with TRIzol reagent (Invitrogen, Grand Island, NY, USA) as previously described [18]. cDNA was synthesized using MMLV reverse transcriptase and random primers. Real-time PCR was conducted using SYBR Green PCR Master Mix (Eurogentec, San Diego, CA, USA). 28S rRNA was used as a loading control. The oligonucleotide primer sequences were as follows: IGF-1, forward, CTG CTT GCT CAC CTT CAC CA, reverse, ATG CTG GAG CCA TAG CCT GT; HGF, forward, GTC AGC ACC ATC AAG GCA AG, reverse, TCC ACGACC AGG AAC AAT GA. Samples were amplified for 35 cycles using an Eppendorf Mastercycler Real-Time PCR machine (Eppendorf, Hamburg, Germany). The mRNA levels in relation to 28S rRNA were determined using a comparative C_T method [18].

2.7. Immunofluorescent staining for stem cells

Three hours after I/R, the heart was perfused with 10% potassium chloride to induce diastolic cardiac arrest followed by saline and 4% paraformaldehyde perfusion for 15 min. After 1 h incubation in 30% sucrose, hearts were embedded in OCT and cut into 5 μm sagittal sections. The sections were stained with primary antibodies (*c-kit*, 1:200, eBioscience, San Diego, CA, USA; Ki67, 1:200, NeoMarkers, Fremont, CA, USA) at 4 °C overnight followed by fluorescent secondary antibody at room temperature for 1 h. CD45 was stained by FITC-conjugated anti-CD45 (1:100, eBioscience, San Diego, CA, USA) at 4 °C overnight. Nuclei were stained with Hoechst 33342. The number of *c-kit*$^+$, Ki67$^+$, CD45$^+$/*c-kit*$^+$ and CD45$^-$/*c-kit*$^+$ cells per sagittal heart section was quantified using a fluorescent microscope (Observer D1, Carl Zeiss Canada, Toronto, Canada). Images were taken using a laser confocal microscope (LSM 510 Meta, Carl Zeiss Canada, Toronto, Canada).

2.8. Isolation and culture of *c-kit*$^+$ CSCs

c-Kit$^+$ CSCs were isolated using a previous protocol [23,24] with modifications. Briefly, hearts from WT mice were isolated and perfused with cold PBS. LV was minced into small pieces and mild digested with 0.05% trypsin–EDTA at 37 °C for 15 min. LV tissue pieces were then transferred to fibronectin coated dish and incubated in complete explant medium (IMDM, 15% fetal bovine serum, 1% penicillin–streptomycin, 1% L-glutamine, and 0.1 mM 2-mercaptoethanol) for 2–3 weeks. Cells grown from the LV tissue pieces were mild digested using 0.05% trypsin-EDTA at room temperature for <3 min. *c-Kit*$^+$ CSCs were pulled-down by *c-kit* antibody (Santa Cruz, CA, USA) coated magnetic beads (Invitrogen, CA, USA) and then cultured in the CSC culture medium (DMEM/F-12, 3.5% FBS, 1% penicillin–streptomycin, 1% L-glutamine, 0.1 mM 2-mercaptoethanol, 80 ng/ml bFGF and 25 ng/ml EGF) for further experiments. To investigate the effects of hSCF overexpression on growth factor expression, *c-kit*$^+$ CSCs were seeded at density of 2×10^4/well of 24-well plates and treated with adenoviral hSCF or LacZ constructs for 24 h. ACK2 (20 μg/ml) was used to block *c-kit* signaling.

2.9. Flow cytometry

The expression of CD45 in isolated CSCs was analyzed using a FACSCalibur flow cytometer (BD Biosciences, Mississauga, ON, Canada). After c-*kit*$^+$ CSCs were pulled-down by *c-kit* antibody (Santa Cruz, CA, USA) coated magnetic beads, the *c-kit*$^+$ and *c-kit*$^-$ cells were stained with fluorescein isothiocyanate-conjugated monoclonal antibody against mouse CD45 (eBIOSCIENCE, San Diego, CA, USA). The percentage of CD45$^+$ cells in *c-kit*$^+$ and *c-kit*$^-$ cells was determined.

2.10. Toluidine blue staining for mast cells

To quantify the mast cells in the heart, cardiac tissue sections were stained with 0.05% toluidine blue (Sigma, St. Louis, MO, USA) in 1% sodium chloride for 2 min. The granules of mast cells show violet as we previously described [18].

2.11. Statistical analysis

Data are expressed as mean ± SEM. Two-way or one-way ANOVA followed by Bonferroni test was performed for multiple group comparisons. Unpaired Student's *t*-test was used for two group comparisons. Differences were considered statistically significant at the level of $P < 0.05$.

3. Results

3.1. Overexpression of hSCF decreases infarct size and myocardial apoptosis after I/R

Forty-five minutes of ischemia followed by 3 h of reperfusion resulted in myocardial infarction in both hSCF/tTA and WT mice. Representative images of heart sections following Evans blue and TTC staining are shown in Fig. 1A. Infarct size of hSCF/tTA mice was significantly decreased to 39% compared to WT mice ($P < 0.05$, Fig. 1B) while areas at risk were similar among all groups (Fig. 1C). Additionally, myocardial apoptosis measured by caspase-3 activity and cell death ELISA in the infarct border zone was significantly decreased in the hSCF/tTA mice compared to WT ($P < 0.05$, Fig. 2). To further verify that the protective effects were due to the hSCF overexpression, hSCF/tTA mice were treated with DOX for two weeks to turn off the cardiac hSCF expression [18] and then subjected to I/R. DOX treatment increased infarct size in hSCF/tTA mice to 52% (Fig. 1B). Myocardial apoptosis in DOX-treated hSCF/tTA mice was also increased to similar levels as the WT groups (Fig. 2).

3.2. Akt signaling contributes to hSCF overexpression-induced cardiac protection after I/R

To evaluate the molecular mechanism responsible for the effects of cardiac-specific hSCF overexpression, PI3K/Akt signaling pathway was studied. Akt phosphorylation was significantly increased in hSCF/tTA mice after I/R compared to WT ($P < 0.05$, Fig. 3A), which was abrogated by DOX treatment. Furthermore, PI3K inhibitor LY294002 also blocked the increase of Akt phosphorylation in hSCF/tTA mice after I/R ($P < 0.05$, Fig. 3B) and resulted in significant increases in myocardial apoptosis (Fig. 2) and infarct size (Fig. 1B). As an important downstream signaling molecule of Akt signaling [5], eNOS phosphorylation was also measured. Similarly, higher levels of eNOS phosphorylation were found after I/R in hSCF/tTA compared to WT mice (Fig. 3C and D).

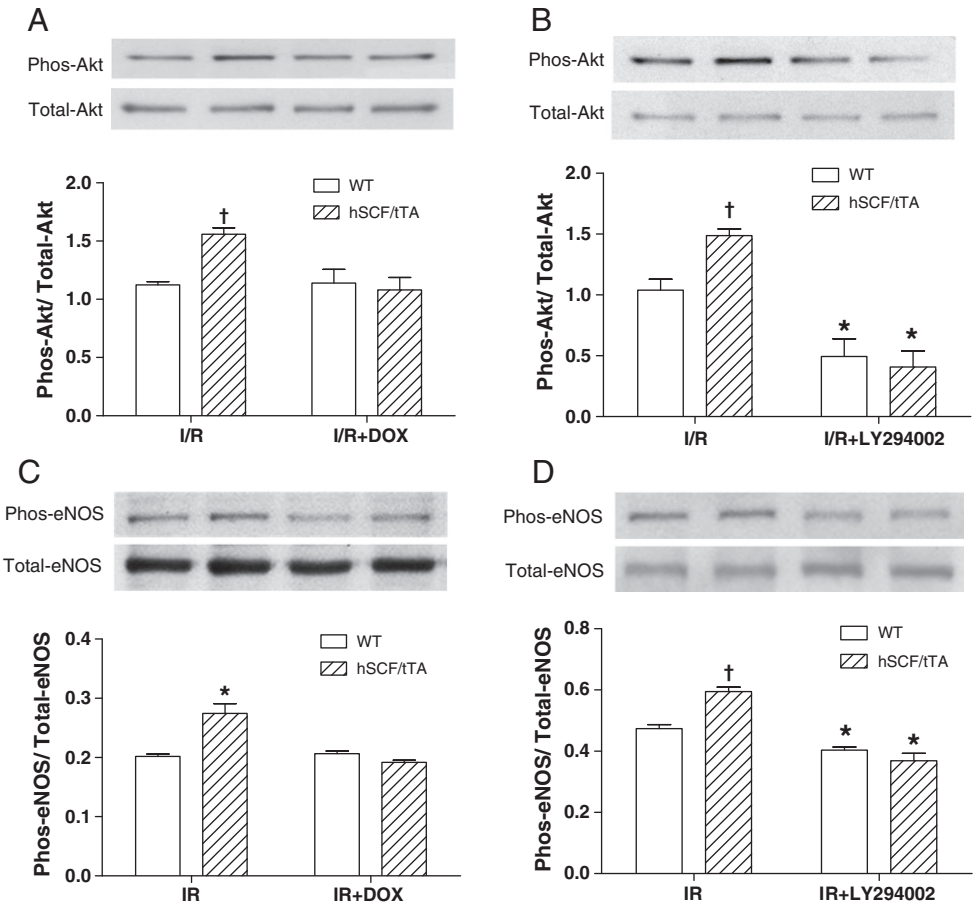

Fig. 3. Myocardial Akt and eNOS phosphorylation in WT and hSCF/tTA mice after I/R. A: Akt phosphorylation was significantly increased in the hSCF/tTA compared to WT mice. DOX treatment abolished this increase in hSCF/tTA mice. B: Inhibition of PI3K significantly lowered Akt phosphorylation both in WT and hSCF/tTA mice. C: Myocardial eNOS phosphorylation was significantly increased in the hSCF/tTA mice compared to WT. DOX treatment abolished this increase in hSCF/tTA mice. D: Inhibition of PI3K significantly impaired eNOS phosphorylation both in WT and hSCF/tTA mice. Data are mean ± SEM, $n = 6$ per group. Two-way ANOVA followed by Bonferroni test: *$P < 0.05$ vs. I/R groups; † $P < 0.01$ vs. WT.

3.3. Overexpression of hSCF increases c-kit$^+$ cells in the myocardium after I/R

c-Kit is the SCF receptor and a surface marker for progenitor/stem cells. Accordingly, *c-kit* immune-fluorescent staining was performed to analyze the number of *c-kit*$^+$ cells in the heart. A representative image of *c-kit*$^+$ cells in the myocardium is shown in Fig. 4A. Two *c-kit*$^+$ cells with large circular nuclei and a thin rim of cytoplasm are located in the interstitial space of the myocardium. In WT sham hearts, the number of *c-kit*$^+$ cells was about 10 cells per heart section while the number was doubled in hSCF/tTA sham hearts ($P < 0.05$, Fig. 4B). After I/R injury, number of *c-kit*$^+$ cells was significantly increased in both WT and hSCF/tTA mice while there were significantly more *c-kit*$^+$ cells in hSCF/tTA compared to WT hearts ($P < 0.05$, Fig. 4B). Consistent with these findings, *c-kit* mRNA levels were significantly increased in both WT and hSCF/tTA mice after I/R. Furthermore, myocardial *c-kit* mRNA levels were significantly higher in hSCF/tTA compared to WT mice after I/R (Fig. 4C). However, cell proliferation in the myocardium after I/R assessed by Ki67 staining did not show any significant difference between WT and hSCF/tTA mice (Fig. 4D). Furthermore, *c-kit* neutralizing antibody ACK2 was used to investigate the role of *c-kit* signaling in the cardioprotection of hSCF overexpression after I/R. Inhibition of *c-kit* function by ACK2 not only abrogated increases in the number of *c-kit*$^+$ cells in the heart (Fig. 4B) but also abolished the reduction of infarct size in hSCF/tTA mice after I/R ($P < 0.05$, Fig. 4E).

3.4. Overexpression of hSCF enhances protective growth factor expression after I/R

Previous studies have implicated growth factors including HGF and IGF-1 in promoting cardiomyocyte survival after I/R [7,25]. Production of these growth factors by stem cells supports the paracrine effects of stem cell therapy in ischemic heart disease [26,27]. To determine the expression levels of these growth factors, real-time RT-PCR and western blot analysis were employed. HGF and IGF-1 mRNA and protein expression were significantly increased after I/R in both WT and hSCF/tTA mice ($P < 0.05$, Fig. 5). However, significantly

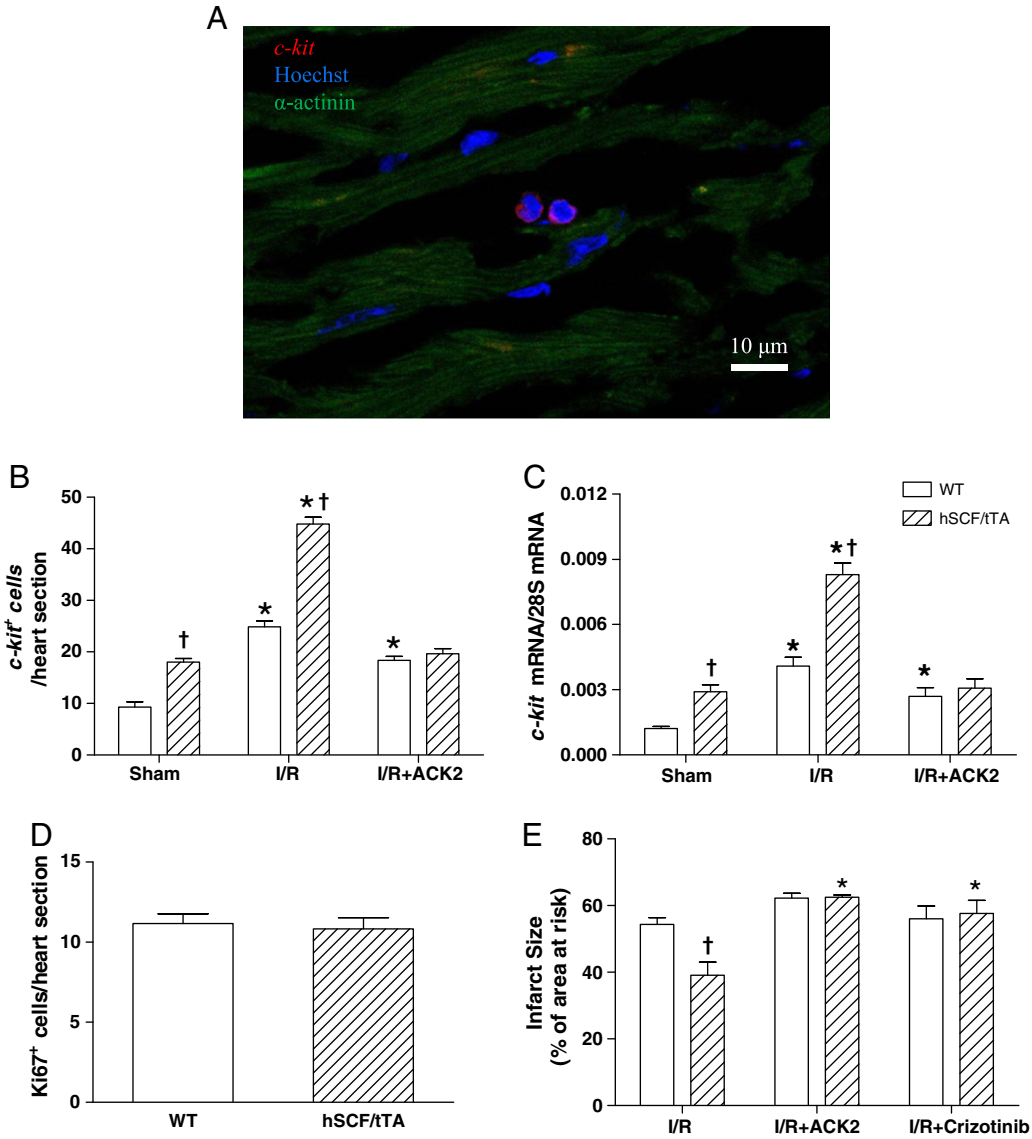

Fig. 4. *c-Kit*$^+$ cells in WT and hSCF/tTA hearts. A: Representative image of *c-kit*$^+$ cells in the myocardium. Hoechst (blue, nuclei), *c-kit* (red) and α-actinin (green). B: Quantitative analysis of *c-kit*$^+$ cells in the heart of WT and hSCF/tTA mice. C: Myocardial *c-kit* mRNA expression was significantly higher in hSCF/tTA compared to WT mice after I/R. Blockade of *c-kit* signaling by ACK2 abrogated this effect. D: Quantitative analysis of Ki67$^+$ cells in the heart of WT and hSCF/tTA mice showed no difference between the two groups (unpaired Student's *t*-test, P = n.s). E: Blockade of *c-kit* by ACK2 and inhibition of HGF receptors by crizotinib abolished the reduction of infarct size in hSCF/tTA mice after I/R. Data are mean ± SEM, n = 5–7. Two-way ANOVA followed by Bonferroni test: $^*P < 0.05$ vs. sham, † $P < 0.05$ vs. WT, $^*P < 0.05$ vs. hSCF/tTA I/R (panel E only).

higher HGF and IGF-1 expression were observed in hSCF/tTA compared to WT hearts ($P < 0.05$, Fig. 5). Blocking *c-kit* signaling by ACK2 abolished the enhanced HGF and IGF-1 expression in the hSCF/tTA heart after I/R ($P < 0.05$, Fig. 5). To further demonstrate the role of HGF in cardioprotection by hSCF overexpression, an inhibitor of HGF receptor crizotinib was used. Treatment with crizotinib (15 mg/kg, IP) abolished the reduction in infarct size after I/R in hSCF/tTA mice with no significant effect in WT mice (Fig. 4E).

3.5. Sub-populations of c-kit$^+$ cells in hearts with hSCF overexpression after I/R

c-Kit is an excellent marker for cardiac progenitors and circulating stem cells [13,28]. However, hematopoietic mast cells (CD45$^+$) are also positive for *c-kit*. In order to further investigate the source of increased *c-kit*$^+$ cells in hSCF/tTA hearts after I/R, CD45 was used to distinguish the *c-kit*$^+$ cell sub-populations. Representative images of CD45$^+$*c-kit*$^+$ and CD45$^-$*c-kit*$^+$ cells were shown in Fig. 6A. CD45$^-$*c-kit*$^+$ cells are cardiac stem cells capable of cardiogenesis [12,13]. Our data show that the overall number of CD45$^-$*c-kit*$^+$ cells in sham and I/R groups was significantly higher in hSCF/tTA hearts compared to WT ($P < 0.05$, Fig. 6B). When the heart was divided into lower and upper halves, increases in CD45$^-$*c-kit*$^+$ cells by hSCF overexpression were more pronounced and mainly occurred in the upper half of the heart ($P < 0.05$, Fig. 6C). Additionally, I/R significantly increased the CD45$^+$*c-kit*$^+$ cell population in the heart compared to sham, which was also enhanced in the hSCF/tTA mice ($P < 0.05$, Fig. 6D). Interestingly, the number of mast cells determined by toluidine blue staining after I/R was similar between WT and hSCF/tTA hearts, suggesting that mast cells do not contribute to the increases of CD45$^+$*c-kit*$^+$ cell population induced by hSCF overexpression (Fig. 6E).

3.6. Overexpression of hSCF induces HGF expression in CSCs in vitro

In order to investigate the effects of hSCF overexpression on growth factor expression, *c-kit*$^+$ CSCs were isolated from the myocardium and cultured *in vitro*. Flow cytometric analysis showed that the isolated *c-kit*$^+$ CSCs were negative for CD45 (Fig. 7A). Treatment with Ad-hSCF significantly increased HGF mRNA levels compared to Ad-LacZ ($P < 0.05$, Fig. 7B). No significant difference in IGF-1 expression was found among all groups (Fig. 7C).

4. Discussion

The present study demonstrated for the first time that cardiomyocyte-specific overexpression of hSCF decreases myocardial apoptosis and infarct size after acute myocardial I/R. When hSCF expression was turned off by DOX treatment, these effects were abrogated, suggesting that overexpression of hSCF is responsible for cardiac protection in I/R. Furthermore, we showed that the cardioprotective effects of hSCF overexpression are due to activation of Akt-PI3K signaling pathway,

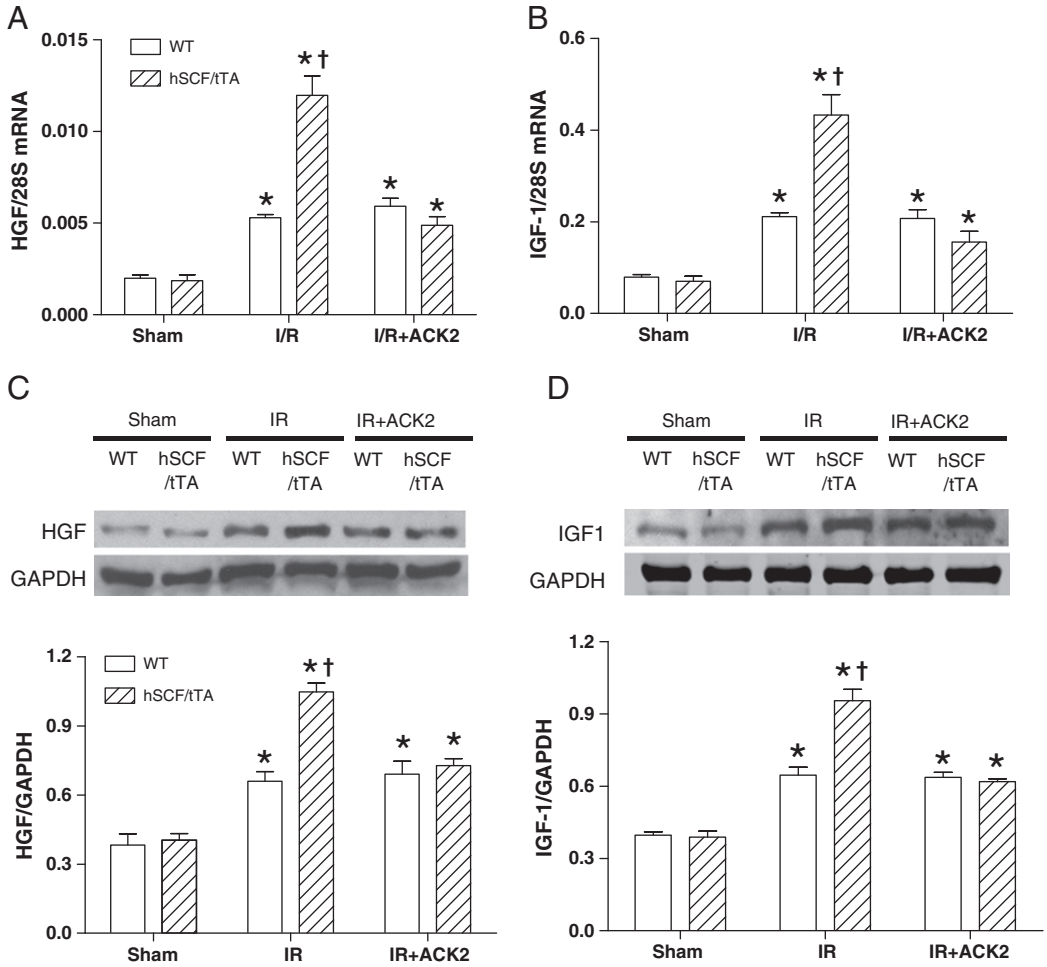

Fig. 5. Cardioprotective cytokine expression in WT and hSCF/tTA mice after I/R. HGF and IGF-1 mRNA and protein levels were determined by real-time RT-PCR (A and B) and western blot analysis (C and D), respectively. After I/R, HGF and IGF-1 expression was significantly increased both in WT and hSCF/tTA mice. However, a significantly higher level of HGF and IGF-1 expression was found in hSCF/tTA compared to WT mice. This increase was abrogated by ACK2 treatment. Data are mean ± SEM, $n = 6$. Two-way ANOVA followed by Bonferroni test: *$P < 0.05$ vs. sham; † $P < 0.05$ vs. WT.

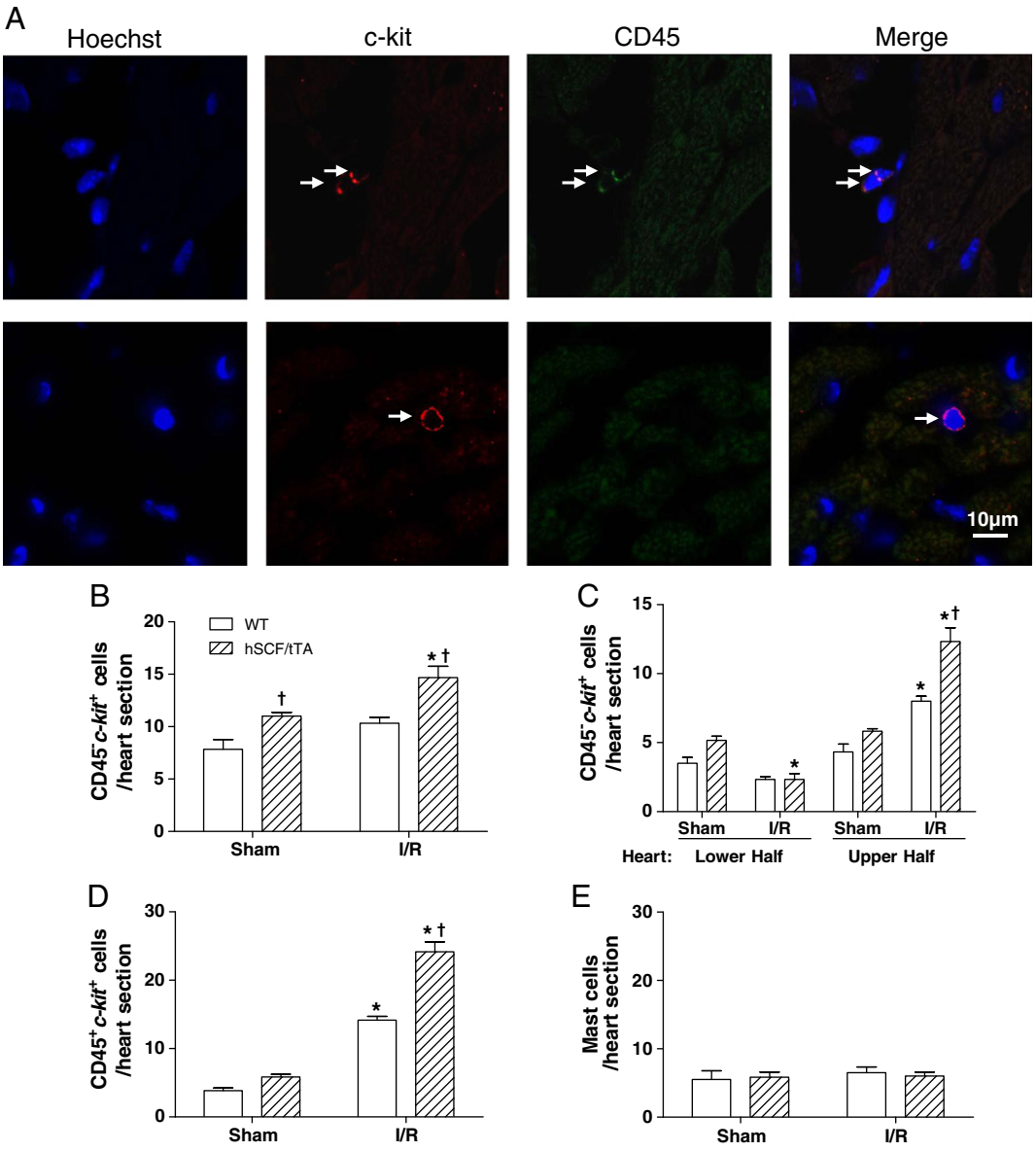

Fig. 6. Sub-population of *c-kit*$^+$ cells in the myocardium after I/R. A: Representative images of CD45$^+$*c-kit*$^+$ cells and a CD45$^-$*c-kit*$^+$ cell in the myocardium. Hoechst (blue, nuclei), *c-kit* (red) and CD45 (green). B: Quantitative analysis shows there were significantly more CD45$^-$*c-kit*$^+$ cells in hSCF/tTA compared to WT hearts in sham and I/R groups. C: CD45$^-$*c-kit*$^+$ cells in the lower and upper halves of the heart in sham and I/R conditions of WT and hSCF/tTA mice. D: CD45$^+$*c-kit*$^+$ cell number was significantly higher in hSCF/tTA mice compared to WT after I/R. E: Quantification of mast cells detected by toluidine blue showed no significant difference between WT and hSCF/tTA mice. Data are mean ± SEM, $n = 6$. Two-way ANOVA followed by Bonferroni test: *$P < 0.05$ vs. sham; † $P < 0.05$ vs. WT.

enhanced growth factor (IGF-1 and HGF) expression and endogenous CSC activation after acute I/R. Our study suggests that hSCF may have therapeutic potential in the treatment of ischemic heart disease.

In acute myocardial I/R, the lack of blood supply during ischemia and the oxidative stress and abrupt metabolic changes immediately after reperfusion lead to severe cellular injuries [29]. It is widely accepted that both necrosis and apoptotic cell death contribute to infarct size development and cell loss during I/R [30]. Apoptosis differs from necrosis in that it is a finely regulated process initiated within the cell [30]. Interventions that suppress myocardial apoptosis during I/R decrease infarct size and improve cardiac function [31,32]. In the present study, we found that cardiac-specific overexpression of hSCF decreased infarct size and myocardial apoptosis after I/R. The fact that reduction in infarct size was completely abolished when hSCF overexpression was turned off further confirmed that the beneficial effects are due to hSCF overexpression in the myocardium. The signaling pathway responsible for the improvement was also investigated. As the major component of the reperfusion injury salvage kinase pathway (RISK) [3], PI3K/Akt/eNOS signaling was increased in hSCF overexpressing hearts after I/R. Blocking the PI3K-Akt signaling by LY294002 abrogated Akt/eNOS activation and increased myocardial apoptosis and infarct size, which clearly demonstrate that PI3K/Akt/eNOS signaling is responsible for the cardiac protection in hearts with hSCF overexpression after I/R.

The activation of RISK pathway can be induced by cardiac protective growth factors during I/R [7]. In this regard, exogenous administration of IGF-1 [33] and HGF [25,34,35] has been shown to decrease myocardial apoptosis and infarct size in acute I/R injury. As an endogenous response, the heart is also able to produce cardioprotective growth factors after ischemia. In our study, acute I/R induced HGF and IGF-1 expression in the WT myocardium, which is consistent with previous studies in the porcine I/R model [36]. This endogenous protection mechanism in acute I/R by releasing beneficial growth factors is further enhanced by hSCF overexpression. Inhibition of HGF receptor signaling abrogated the improved infarct size in hSCF overexpressing mice after I/R, suggesting that HGF contributes to the protective effects of hSCF

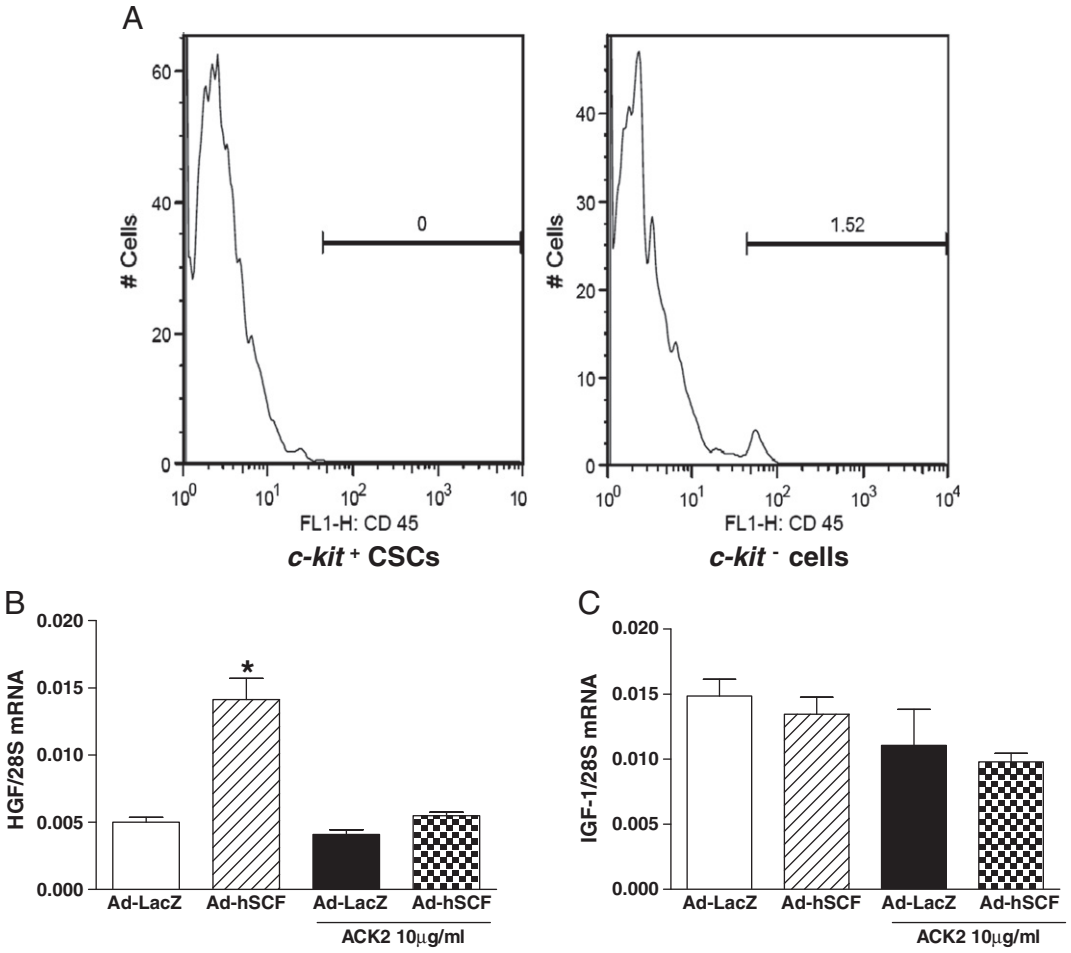

Fig. 7. Effects of hSCF overexpression on growth factor expression in cultured *c-kit*$^+$ CSCs. A: Flow cytometric analysis showed the cultured *c-kit*$^+$ CSCs were negative for CD45 while 1.5% of *c-kit*$^-$ cells were positive for CD45. B: Adenoviral hSCF overexpression in *c-kit*$^+$ CSCs induced HGF mRNA expression, which was abrogated by ACK2 treatment. C: IGF-1 mRNA expression was not significantly different among all groups. Data are mean ± SEM, $n = 3$; One-way ANOVA followed by Bonferroni test: $^*P < 0.05$ vs. all other groups.

overexpression. More importantly, our study provided convincing evidence that SCF/*c-kit* signaling plays a critical role in this process as the increased expression of HGF and IGF-1 was abolished by ACK2 treatment in mice with hSCF overexpression.

It is now recognized that mechanisms of cardiac protection from endogenous stem/progenitor cell recruitment and/or exogenous stem cell delivery may include the paracrine effects [37], which result from the secretion of various cytokines, chemokines and growth factors [38,39], and possibly direct differentiation into cardiomyocytes [40] and vascular cells [18]. Despite significant beneficial effects observed in animal models, clinical trials using bone marrow stem cells show only minor improvements in MI patients [41]. The key issues that need to be resolved include choosing the optimal stem cell type [28,42], and safe and efficient delivery method [40] and maximizing cell retention and engraftment [43]. Mobilization of hematopoietic and endothelial progenitors to the peripheral blood occurs within a few hours after acute MI, acting as an endogenous response to tissue damage [44]. Our efforts have been focused on the enhancement of the endogenous recruitment and retention of the stem/progenitor cells to the ischemic myocardium. We recently showed that hSCF overexpression increases the number of EPCs in the myocardium 5 days post-MI [18]. In the present study, a significant increase of *c-kit*$^+$ cell numbers in the WT myocardium after acute I/R compared to sham was observed, which is an endogenous response to myocardial ischemia. By overexpressing hSCF in cardiomyocytes, we showed for the first time that *c-kit*$^+$ cell population was further increased after acute I/R compared to WT. Furthermore, when the SCF/*c-kit* signaling was blocked by ACK2, the increase of *c-kit*$^+$ cells induced by hSCF overexpression after I/R was abrogated, illustrating a critical role of SCF/*c-kit* interaction in this process.

The *c-kit*$^+$ cells in the adult heart have two sub-populations, CD45$^-$ *c-kit*$^+$ and CD45$^+$*c-kit*$^+$ cells. The CD45$^-$*c-kit*$^+$ cells are cardiac stem cells (CSCs) which are able to differentiate into cardiomyocytes [12,40,45,46]. Studies have shown that the number of CSCs is increased 6 days after MI [47]. In the present study we showed that the number of CSCs was increased in the upper half of the heart, which was mainly non-infarct area of the myocardium after I/R, and was further enhanced by hSCF overexpression. The increase in *c-kit*$^+$ cell population induced by hSCF overexpression is possibly due to cell migration and recruitment from CSC niches or the circulation since cell proliferation assessed by Ki67 staining did not show a significant difference between WT and hSCF overexpressing mice after I/R. Interestingly, under basal conditions, more CSCs were found in hSCF overexpressing myocardium compared to the WT, which suggests the pivotal role of membrane-associated SCF in maintaining a CSC pool in the myocardium of the adult heart. Despite increases in number, CSCs in the hSCF overexpressing heart remain quiescent until an ischemic insult occurs, which is confirmed by the finding that the expression of HGF and IGF-1 is not increased in hSCF overexpressing mice under basal conditions. The effects of hSCF on CSCs activity were further studied in isolated *c-kit*$^+$ CSCs *in vitro*. Overexpression of hSCF induced HGF expression in CSCs via *c-kit* signaling.

Our study also showed a significant increase in CD45$^+$*c-kit*$^+$ cells in the heart after I/R. Moreover, hSCF overexpression resulted in 1.7 fold increase in CD45$^+$*c-kit*$^+$ cells compared to WT mice after I/R.

The $CD45^+c\text{-}kit^+$ cells are of hematopoietic lineage which includes mast cells, circulating hematopoietic stem cells [48] and endothelial progenitors [49]. We first confirmed that the increase of $CD45^+c\text{-}kit^+$ cells was not due to mast cells since the number of mast cells was not significantly altered in WT and hSCF overexpressing mice after I/R. Thus, the increases in the number of $CD45^+c\text{-}kit^+$ cells suggest that hSCF overexpression promotes recruitment of circulating progenitor cells after I/R. These data are consistent with previous findings from our lab and others that interaction of membrane-associated SCF and *c-kit* enhances circulating $c\text{-}kit^+$ progenitor recruitment [18,50].

In conclusion, cardiomyocyte-specific hSCF overexpression decreases infarct size and myocardial apoptosis after acute I/R. These beneficial effects are likely due to enhanced activation of PI3K/Akt/eNOS signaling and production of cardioprotective growth factors induced by increased population of $c\text{-}kit^+$ progenitors in the ischemic myocardium. The ability of membrane-associated SCF to provide cardioprotection in both acute I/R and chronic heart failure [18] makes it a promising candidate as a new treatment for ischemic heart disease.

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