Mol. Cells *29*, 9-19, January 31, 2010
DOI/10.1007/s10059-010-0001-7

Molecules and Cells

Mesenchymal Stem Cells Modified with Stromal Cell-Derived Factor 1α Improve Cardiac Remodeling via Paracrine Activation of Hepatocyte Growth Factor in a Rat Model of Myocardial Infarction

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Mesenchymal stem cells (MSCs) are a promising source for cell-based treatment of myocardial infarction (MI), but existing strategies are restricted by low cell survival and engraftment. We examined whether SDF-1 transfection improve MSC viability and paracrine action in infarcted hearts. We found SDF-1-modified MSCs effectively expressed SDF-1 for at least 21days after exposure to hypoxia. The apoptosis of Ad-SDF-1-MSCs was 42% of that seen in Ad-EGFP-MSCs and 53% of untreated MSCs. In the infarcted hearts, the number of DAPI-labeling cells in the Ad-SDF-1-MSC group was 5-fold that in the Ad-EGFP-MSC group. Importantly, expression of antifibrotic factor, HGF, was detected in cultured MSCs, and HGF expression levels were higher in Ad-SDF-MSC-treated hearts, compared with Ad-EGFP-MSC or control hearts. Compared with the control group, Ad-SDF-MSC transplantation significantly decreased the expression of collagens I and III and matrix metalloproteinase 2 and 9, but heart function was improved in d-SDF-MSC-treated animals. In conclusion, SDF-1–modified MSCs enhanced the tolerance of engrafted MSCs to hypoxic injury in vitro and improved their viability in infarcted hearts, thus helping preserve the contractile function and attenuate left ventricle (LV) remodeling, and this may be at least partly mediated by enhanced paracrine signaling from MSCs via antifibrotic factors such as HGF.

INTRODUCTION

Mesenchymal stem cells (MSCs) are pluripotent, adult stem cells and mostly reside within the bone marrow. MSCs can be obtained in relatively large numbers through standard clinical procedures, and MSCs are easily expanded in culture. Animal studies and early clinical experience showed that therapeutically-delivered MSCs safely improve heart function after an acute myocardial infarction (MI) (Shao et al., 2008). The effects of transplanted MSCs could the result of the potential of MSCs to differentiate into cardiomyocytes, endothelial cells, and vascular smooth muscle cells *in vitro* and *in vivo*. However, recent studies have shown that transplanted MSCs repair infarcted hearts through their paracrine actions, such as secreted growth factors and angiogenic factors (Fedak et al., 2008). Unfortunately, limited cell viability under hypoxic conditions restricts the optimal effects of their capacity.

Stromal cell-derived factor 1α (SDF-1α or CXCL12) is a major chemotactic factor for stem cells (Schober et al., 2006). Overexpression of SDF-1α by adenoviral gene delivery and SDF-1α protein enhanced the recruitment of stem cells [e.g hemopoietic stem cells (HSC), MSCs, cardiac stem cells (CSCs), and endothelial progenitor cells (EPCs)] to the infarcted heart. In addition, SDF-1 is cardioprotective after MI. These findings showed that SDF-1 has multiple effects on myocardial structure and function via cardioprotection, myogenesis, angiogenesis and anti-inflammation (Elmadbouh et al., 2006; Saxena et al., 2008; Schuh et al., 2008; Tang et al., 2008; Yamaguchi et al., 2003). Overexpressing-SDF-1α MSCs could repair the infarcted heart though the nutrition effects of SDF-1α on cardiocytes (Yang et al., 2006). Interestingly, several studies have been demonstrated that SDF-1 could enhance the survival of MSCs via the activation of Akt (Li et al., 2007; Zhang et al., 2007).

We therefore hypothesized that SDF-1 transfection might help the survival of transplanted MSCs, which might also help sustainable release of paracrine factors other than SDF-1. The purpose of this study was to assess the *in vitro* effects of hypoxia on the survival of SDF-1-modified MSCs. To this end, the left anterior descending coronary artery (LAD) was ligated to produce a rat model of myocardial infarction (MI) allowing us to examine the effects of SDF-1-modified MSCs on ventricular remodeling and cardiac function.

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Received July 9, 2009; revised September 10, 2009; accepted September 25, 2009; published online December 10, 2009

Keywords: angiogenesis, HGF, myocardial infarction, remodeling, SDF-1α, stem cell

MATERIALS AND METHODS

Isolation and culture of MSCs

Isolation and primary culture of MSCs has been described elsewhere (Tang et al., 2006). Briefly, rat MSCs were isolated from bone marrow by density gradient centrifugation. They were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal calf serum (FCS; Hyclone, Co), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was removed and replaced with fresh medium twice a week. At confluence, cells were harvested for passage with 0.25% trypsin (USA) containing 0.02% ethylene diamine tetra-acetic acid (EDTA) (Sigma, USA). Experiments were carried out using cells from the fourth passage.

Characterization of SDF-1-modified MSCs after hypoxia exposure

Ad-SDF-1, an adenoviral vector containing the human SDF-1α gene under the control of the cytomegalovirus (CMV) promoter, and Ad-EGFP, an adenoviral vector containing the nuclear targeted green fluorescence protein gene EGFP under the control of the CMV promoter, have been used in previous studies from our laboratory (Tang et al., 2008). Virus titration was determined by TCID50 assay, the titer of which was approximately 1.4×10^{12} Pfu/ml.

The transduction of MSCs with adenovirus was carried out as previously described (Tang et al., 2009a). Briefly, to select the best multiplicity of infection (MOI) for adenovirus-mediated gene transfer, MSCs were exposed to Ad.EGFP at MOI 10, 50, 100, 150, and 200 for 12 h. Fluorescence microscopy was used to observe cell viability, focusing on to cell morphology. FACS was used to quantify the transfection efficiency of the adopted MOI according to the expression of EGFP. The optimal MOI in the following experiments was chosen for both the highest EGFP expression and viability.

The protein expression of Ad.SDF-1-MSC, Ad.EGFP-MSC, and non-treated MSCs was determined by Western blot analysis. Two days after transfection, MSCs were homogenized to remove nuclei and cell debris and the protein was extracted. After SDS-gel electrophoresis using 12% polyacrylamide gels, the proteins were probed by goat anti-human SDF-1 antibody (Santa Cruz Biotech, Inc.).

MSCs were transfected with adenoviral vectors (Ad-SDF-1, Ad-EGFP) at the optimal MOI. The cells were incubated overnight under normoxic conditions (21% O_2, 25% CO_2, and balance N_2), and the media were replaced. For normalization purposes, the cells were plated in 12-well plates (Corning) in a concentration of 1×10^5 cell/well/ml in complete medium for 12 h. Then cells were exposed to hypoxia (1.0% O_2) for 10 h twice a day. The supernatants were collected at 1, 2, 3, 5, 7, 9, 11, 15, and 21 days after treatment.

The levels of secreted hSDF-1 in the supernatants were quantified by ELISA using a human commercially available kit (R&D Systems Inc.) according to the manufacturer's instructions. All samples and standards were measured in duplicate. At the same time, the levels of rat SDF-1 in the supernatants were tested by ELISA (R&D Systems Inc.).

Cell apoptosis and death assays *in vitro*

Cell apoptosis was assayed using terminal deoxynucleotidyl transferase biotin-dUPT nick end labeling (TUNEL). In brief, a TUNEL staining kit (Dead End Fluorometric TUNEL system, Promega, USA) was used to visualize cell death in sections. After 10 min of fixing by 4% paraformaldehyde and pretreatment at -20°C with ethanol/acetic acid (2:1) and 0.2% Triton X-100, the sections were incubated in an equilibration buffer. The TdT enzyme and nucleotide mix was then added at proportions specified by the kit for 75 min at room temperature. Slides were washed with 2× standard saline citrate for 15 min and followed by three washings with PBS. Cell death was assayed using trypan blue staining as described previously (Hu et al., 2009).

Myocardial ischemia model and cell implantation

MI was achieved by ligation of the LAD as previously described (Tang et al., 2008). Briefly, male Wistar inbred rats were anesthetized with ketamine (50 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Tracheal ventilation was carried out with room air using a Colombus ventilator (HX-300, Taimeng Instruments, Chendu, China). After left lateral thoracotomy in the fourth intercostal space, the LAD was ligated. Before chest closure, infarction was confirmed by observation of injury demarcation using blanching of the myocardium and electrocardiography. The rats were randomized into four groups. Five days after MI induction, 0.5×10^6 Ad-EGFP-MSC and 0.5×10^6 Ad-SDF-MSC suspensions in 0.2 mL of serum-free medium were injected separately into four sites (0.05 ml per site) for each MI heart in the transplantation group with a 30-gauge tuberculin syringe. Two injection sites were in the myocardium bordering the infarcted area and two were within the infarcted area. The control group received identical MI surgery, but only an equivalent volume of cell-free medium was injected. Penicillin (150,000 U/ml, i.v.) was given before each operation and buprenorphine hydrochloride (0.05 mg/kg, s.c.) was administered twice a day for the first 48 h after surgery.

RNA preparation and RT-PCR

Total RNA was extracted from cardiac muscle tissue or cultured MSCs using TRIzol Reagent (Invitrogen, USA) following the manufacturer's protocol. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the Moloney Murine Leukemia Virus reverse transcriptase (Fermentas Life Science, Canada) and oligo (dT) primers according to the manufacturer's instructions. The primer sequences and reaction conditions were as follows:

MMP-2(761bp): Forward 5′GGCCATGCCATGGGGCTGGA3′, Reverse 5′CCAGTCTGATTTGATGCTTC3′, MMP-9 (234 bp): Forward 5′AACCCTGGTCACCGGACTTC3′, Reverse 5′CACCCGGTTGTGGAAACTCAC3′), HGF (337 bp): Forward 5′CTGTCACCATCCCCTATG3′, Reverse 5′CTGCCTCCTTTACCAATG-3′, hSDF-1α (270 bp): Forward 5′CATGAACGCCAAGGTCGTG3′, Reverse-5′TCCAGGTACTCCTGAATCC3′, Collagen I (462 bp): Forward 5′TGCCGTGACCTCAAGATGTG3′, Reverse 5′CACAAGCGTGCTGTAGGTGA3′, Collagen III (336 bp): Forward 5′CGAGGTGACAGAGGTGAAAGA3′, Reverse 5′AACCCAGTATTCTCCGCTCTT3′ and GAPDH (623 bp) Forward 5′CCAAAAGGGTCATCATCTCC3′, Reverse 5′GTAGGCCATGAGGTCCACCAC3′.

PCR was performed at 95°C for 4 min, followed by 25-33 cycles of 94°C for 45 s, 56-58°C for 40 s, and 72°C for 40 s. The constitutively expressed gene GAPDH was used as an internal control for the amount of input cDNA. 20 μl of each PCR product was run on a 1.5% agarose gels. Densitometry was used to quantify amplicon amounts from cardiac tissue or cells.

Western blotting

Western blotting was done with rabbit polyclonal antibody raised against HGF and MMP2 (1:500, Santa Cruz), and goat polyclonal antibody raised against MMP9 (1:500, Santa Cruz) to identify protein expression of HGF, MMP2, and MMP9. The

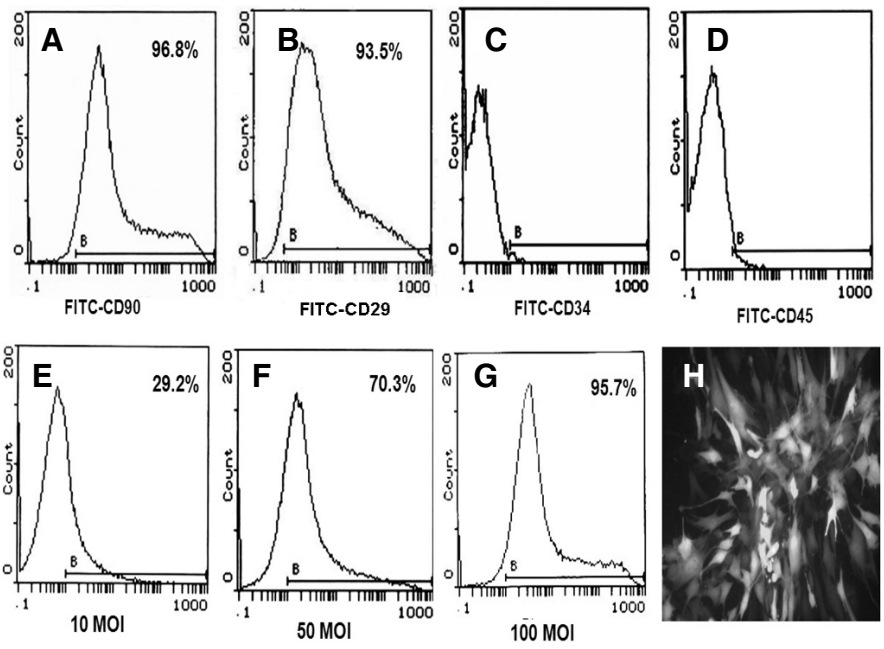

Fig. 1. (A-D) Phenotype characterization of MSCs. Flow cytometric analysis showed that MSCs expressed CD90 (96.5%), CD29 (93.5%), CD34 (0.67%), and CD45 (1.36%). (E-H) Ability of the adenoviral vector to infect MSCs. MSCs were transfected with EGFP recombinant adenoviruses and the percentage of MSCs expressing EGFP was analyzed via FCM. The percentage of transfected cells increased rapidly between MOI 10 and 50 (E, F). At MOI 100, the percentage of EGFP positive cells reached a maximum (G) and were maintained morphologically as fibroblast-shaped cells (H, 200×).

left ventricle (LV) was obtained from individual rats and used for comparisons among all four groups seven days after treatment (each group, n = 5). These samples were homogenized on ice in 0.1% Tween20 homogenization buffer with a protease inhibitor. 60 μg of protein was transferred into sample buffer, loaded on a 7.5% sodium dodecyl sulfate-polyacrylamide gel, and blotted onto a polyvinylidene fluoride membrane (Millipore Company). After being blocked for 120 min, the membrane was incubated with primary antibody (1:250 dilution). After extensive washing, immunocomplexes were detected with horseradish peroxidase-conjugated appropriate secondary antibodies (anti-rabbit IgG, 1:10000 dilution, anti-goat IgG, Santa Cruz) followed by enhanced chemiluminescence reaction (Amersham pharmacia biotech, America) and measured by densitometry (Tang et al., 2008).

Measurement of hemodynamics

Twenty-eight days after treatment, hemodynamic measurements *in vivo* were made. Rats were anesthetized with pentobarbital sodium (60 mg/ kg, i.p.). The carotid artery and femoral artery were isolated. Two catheters were filled with heparinized (10 U/ml) saline solution and connected to a Statham pressure transducer (Gould, USA). The carotid arterial catheter was advanced into the left ventricle to record ventricular pressure. The femoral artery catheter was inserted into an isolated femoral artery to monitor mean arterial pressure (BP) and heart rate (HR). These hemodynamic parameters were monitored simultaneously and recorded on a thermal pen-writing recorder (RJG-4122, Nihon Kohden, Japan) and on an FM magnetic tape recorder (RM-7000, Sony, Japan). The hearts were rapidly removed from killed rats (each group, n = 10-12) after the measurements were taken (Tang et al., 2006).

Histology and morphometric measurement

At day-28 after treatment, the wet weight of rats was measured. left ventricle function was assessed. Hearts were quickly removed and cut into six transverse slices (apex to base) and processed for histology. Briefly, left ventricles were sliced transversely into 5-6 sections (thickness, 2 mm) and incubated for 30 min in a 1% solution of buffered triphenyltetrazolium chloride (TTC) at 37°C. Slices were photographed with a digital camera with magnification to identify the infarcted myocardium (unstained by TTC) and the non-ischemic zones (stained brick-red). Two experienced technicians who were blinded to the study protocol did all the measurements for the treatment group. left ventricle-wall thickness was measured at three widely spaced locations within the scar segment as well as in the non-infarcted region for each slide and the mean value calculated. To estimate the overall degree of ventricular remodeling, the expansion index was determined on day-28 using 10% paraformaldehyde-fixed tissue as previously described (Tang et al., 2006). The expansion index was calculated using the following formula: expansion index = (left ventricle cavity area/total left ventricle area) × (septal thickness/scar thickness). Analyses of left ventricle-wall thickness and the expansion index were done on two middle slides and the mean values calculated for each heart (each group, n = 6) (Koch et al., 2006).

Immunohistochemistry analysis

Rats were killed 7 or 28 days after treatment (n = 5 at each time point). After quick removal, the hearts were immersion-fixed in 4% paraformaldehyde and embedded in optimum cutting temperature (OCT) compound (Fisher Scientific, Fair Lawn). Serial transverse sections of 5 μm length were cut across the entire long axis of the heart and mounted on slides. To block endogenous peroxide activity and nonspecific binding, sections were incubated with 3% hydrogen peroxide followed by 10% normal horse serum. Specimens were incubated with an anti-cTnt (1:250, NeoMarkers) antibody at 4°C overnight. Positive stains were shown as red fluorescence with tetramethyl rhodamine isothiocyanate (TRITC) IgG.

Specimens were also stained with anti-vw VIII factor antibody to confirm capillary density. Five fields from three sections were randomly selected from each sample, and the number of capillaries were counted manually by two pathologists unaware of the rat grouping. Capillary density was calculated as the mean number of capillaries per high-power field (mm^2). Transplanted MSCs labeled with DAPI were identified in tissue sections by

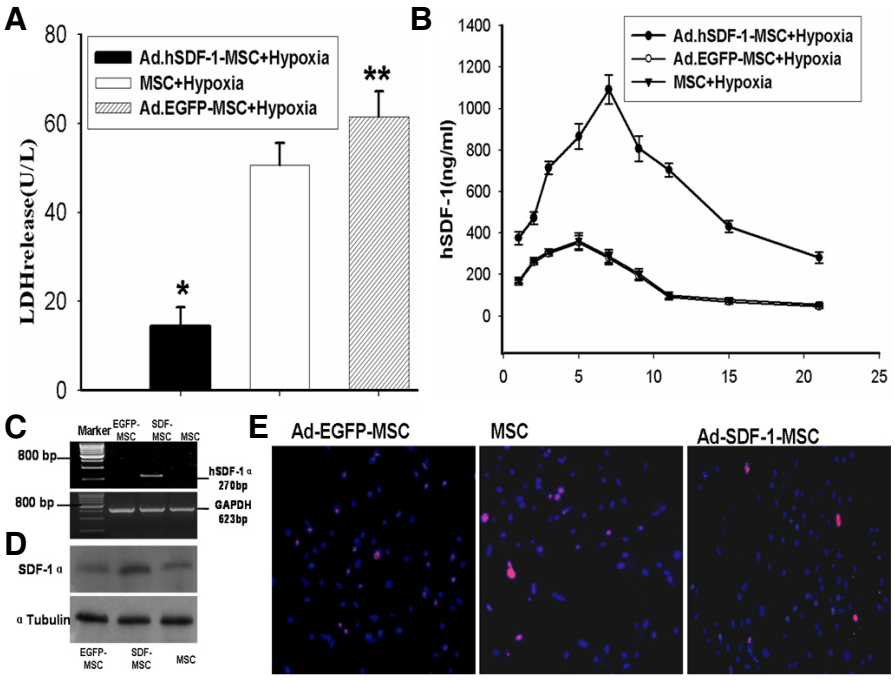

Fig. 2. Effect of hypoxia on hSDF-1α modified MSCs. (A) Under low oxygen tensions, LDH release from MSCs was significantly lower in the Ad-SDF-1-MSC group compared to the MSC or Ad-EGFP-MSC groups. n = 5 per group and $^{*}P < 0.001$ vs. the other groups, $^{**}P < 0.05$ vs. MSC group. (B) Culture media collected at different time points were examined for SDF-1 secretion. There was less than 180 pg/ml in EGFP-transfected MSCs or untransfected MSCs culture medium, but levels reached 1100 pg/ml in the SDF-1 transfected MSCs culture medium at 7 days and remained at a high level over a 21-day period. n = 5 per group and $^{*}P < 0.001$ vs. the other groups, $^{**}P > 0.05$ vs. MSC group. (C) Twenty-one days after hypoxia, hSDF-1α mRNA was detected in the Ad-SDF-1-MSC group, but not in the Ad-EGFP-MSC or untransfected MSC groups. GAPDH served as an internal control. (D) Western blot revealed specific bands in each group 21 days later, but SDF-1 protein was highly expressed in the Ad-SDF-1-MSC group compared to the Ad-EGFP-MSC or MSC groups. N = 5, α-tubulin served as an internal control. (E) SDF-1 significantly decreased cell apoptosis by TUNEL assay.

blue fluorescence. The number of DAPI-labeled MSCs and cTnt-positive myocardium density was counted in a similar way to that for capillary density.

Collagen volume fraction (CVF)

To analyze collagen accumulation, Masson's trichrome staining was done to delineate collagen content as a percentage of the infarction and peri-infarction areas. Sixteen areas of high-power fields (200×) in each section were visualized under light microscopy and photographed at the same exposure time. The CVF in the infarction and peri-infarction areas was calculated as the percentage of stained tissue in the sum of the muscle area and connective tissue by a densitometric method using Image Pro5.02 software (each group, n = 6) (Koch et al., 2006).

Data analysis

The data are expressed as means ± SEM. Statistical significance between two groups was determined by paired or unpaired Student's *t*-test. Results for more than two experimental groups were evaluated by one-way ANOVA to specify differences between groups. $P < 0.05$ was considered significantly different.

RESULTS

Phenotype characterization of MSCs

To characterize the phenotype of MSCs, the expression surface molecules were analyzed. Almost all cultured cells expressed CD29 and CD90, though they did not express CD34 and 45 (Fig. 1). These characteristics were similar to what was previously described for MSCs (Pittenger et al., 1999).

Adenoviral vector transfection of MSCs

The ability of the adenoviral vector to infect MSCs was tested with an AdEGFP vector. The cytopathic effect was obvious in MSCs at MOI 200. MSCs became round-shaped and detached from the wall in 3 days, and no more than 50% of MSCs were alive after 7 days (Fig. 1). However, adenovirus-mediated EGFP gene transduction did not influence the growth characteristics of MSCs at MOI 10, 50, 100, 150, and 200. The transduction efficiency, as determined by the percentage of EGFP positive cells, was 21.37 ± 1.58% at MOI 10, 79.65 ± 5.41% at MOI 50, and 95.74 ± 3.01% at MOI 100.The highest efficiency was found to be at MOI 100 ($P < 0.05$ compared to both MOI 10 and 50 (Fig. 1), and the MSCs were a morphologically homogenous population of fibroblast-shaped cells (Fig.1). Because of their high efficiency and low toxicity, the adenoviral vectors were used to infect MSCs at MOI 100 in the following experiments.

Characterization of hSDF-1α modified MSCs

LDH release from cells after hypoxia was used as an indicator of cellular injury. Compared with the Ad-EGFP-MSC group, the Ad-SDF-1-MSC group showed the highest level of resistance to hypoxia, while the Ad-EGFP-MSC group showed the highest sensitivity. LDH levels were lowest in the Ad-SDF-1-MSC group, compared with Ad-EGFP-MSC or MSC groups (Fig. 2A). To quantify the secretion of SDF-1 from Ad-SDF-1-MSC after hypoxia, the cells were cultured for 21 days. The SDF-1 protein level in the medium cultured with Ad-SDF-1-MSCs was significantly greater than that in the control medium cultured with MSCs or Ad-EGFP-MSCs at each time point, and reached a peak level 7 days after transfection (Fig. 2B). As shown in Figs. 2C and 2D, SDF-1 mRNA and protein were highly expressed in Ad-hSDF-MSCs. Cell apoptosis under hypoxia was measured by TUNEL. The apoptosis of Ad-SDF-1-MSCs was 42% that of Ad-EGFP-MSCs and 53% of untreated MSCs (Fig. 2E).

hSDF-1α modified MSCs survival and SDF-1α expression in the transplanted area

To confirm MSC survival in the implanted area, cultured MSCs

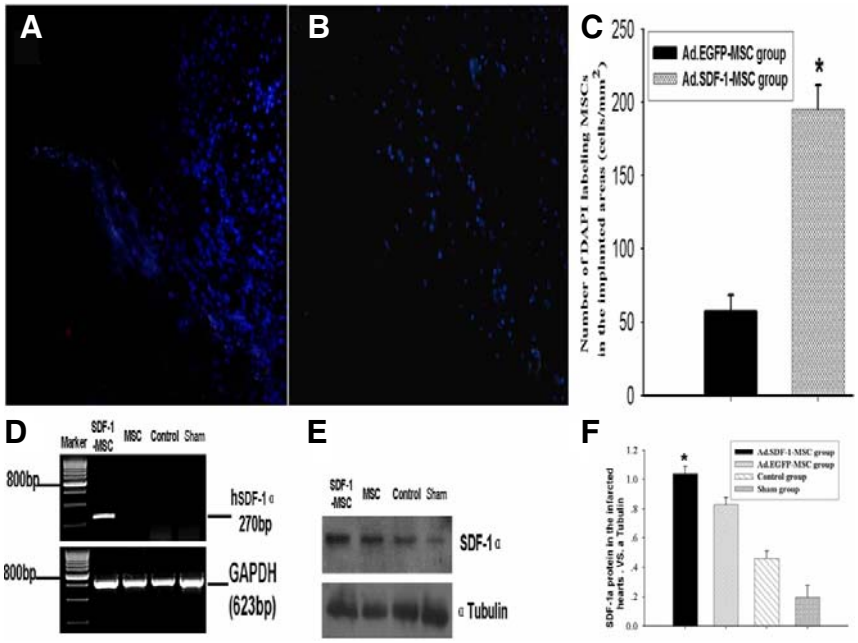

Fig. 3. hSDF-1α modified MSCs survival and SDF-1α expression in the transplanted area. (A, B) Representative sections of surviving MSCs transfected with Ad-SDF-1 in infarcted hearts. (C) Semi-quantitative assay of survival rate of MSCs in infarcted hearts. (D) hSDF-1 expression in the implanted areas was detected by RT-PCR. (E) Western blotting assay of expression of SDF-1 protein in the implanted areas. α-tubulin served as the internal control. (F) Semi-quantitative assay of SDF-1 protein expression by optical density value. n = 5 per group and $*P < 0.001$ vs. the other groups.

were labeled with DAPI prior to transplantation, the labeling efficiency being 100%. One week after cell implantation, the number of DAPI labeling cells of the Ad-SDF-1-MSC group were 5-fold that of the Ad-EGFP MSC group (Figs. 3A-3D). At the same time, hSDF-1 mRNA expression in the implanted area was further quantified using RT-PCR (Fig. 3D). Furthermore, the SDF-1 protein level in the implanted area of animals treated with Ad-SDF-1-MSCs was higher than that of the other groups (Figs. 3E-3F). However, we were unable to detect hSDF-1 mRNA expression in the control and sham groups at 28 days.

Effects of SDF-1 modified MSCs transplantation on angiogenesis

Immunofluorescence demonstrated that partly transplanted MSCs were positive for the cardiac marker cardiac troponin-T, suggesting that a small number of transplanted MSCs had characteristics of cardiomyocytes (Figs. 4A-4C). The efficiency of differentiation of injected Ad-SDF-1-MSCs was higher than in the other groups. The efficiency of differentiation of total MSCs was < 5% per 200× field. The area and number of cardiomyocytes within the infarct zone increased 3.6-fold in the Ad-SDF-1-MSC group compared to the control and Ad-EGFP-MSC groups.

The angiogenic effect of SDF-1 overexpression was measured in terms of blood vessel density in the infarct regions after immunostaining for vWF-VIII (Fig. 4). The numbers of blood vessels per area was higher in the Ad-EGFP MSC group ($p < 0.001$) compared with the control and Ad-EGFP MSC groups (Fig. 4). Immunohistochemical staining further confirmed that the effects of angiogenesis were enhanced. As shown in Fig. 4, a higher capillary density was observed in the sections stained for vWF-VIII factor in the peri-infarct area of the Ad-SDF-1-MSC rats compared to the Ad-EGFP MSC and control groups.

Effects of SDF-1 modified MSCs transplantation on heart fibrosis

Representative Masson's trichrome-stained sections showed more collagen accumulation in the infarcted area and peri-infarction area 28 day after MI in the control and Ad.EGFP-MSC groups than in the Ad.SDF-1-MSC group (Figs. 5A-5D). Semi-quantitative analysis indicated that the collagen volume fraction in the infarcted area was significantly decreased in the Ad.EGFP-MSC group compared to control and Ad.EGFP-MSC groups (Fig. 5E). RT-PCR assay showed the decreased expression of type I and III collagen in the infarcted hearts of the Ad.SDF-1-MSC group compared to the other groups (Figs. 5F and 5G).

Effects of SDF-1 modified MSCs transplantation on MMP expression

Both MMP-2 and MMP-9 mRNA were significantly increased in the control group compared to the sham group. Interestingly, however, the increases in MMP-2 mRNA and MMP-9 mRNA levels were attenuated markedly by Ad.SDF-1-MSC or Ad. EGFP-MSC transplantation (Figs. 6A and 6B). In addition, Ad.SDF-1-MSC or Ad.EGFP-MSC transplantation led to significant lower MMP-2 and MMP-9 protein levels, compared to the control group (Figs. 6D and 6E). However, MMP-2 and MMP-9 expression were slightly but non-significantly increased in the Ad.SDF-1-MSC group compared to the Ad.EGFP-MSC group.

Paracrine signaling by MSCs

Hepatocyte growth factor (HGF) is an important antifibrotic factor. Expression of HGF mRNA was detected in MSCs (Fig. 6C) and there was a significant difference in cultured MSCs, Ad.EGFP-MSCs, and Ad.SDF-1-MSCs under normoxic by RT-PCR and ELISA (data not shown). Furthermore, *in vitro*, the levels of secreted HGF in the supernatants of the Ad.SDF-1-MSC group (586 ± 27 pg/ml) were clearly greater than the MSCs (238 ± 15 pg/ml) and Ad.EGFP-MSCs (217 ± 13 pg/ml) groups,when the cells were exposed to hypoxia (1.0% O_2) for 24 h, as assessed by ELISA. Interestingly, RT-PCR analysis showed that HGF expression in the myocardium was clearly upregulated after MI. Furthermore, the upregulation of HGF expression in the myocardium of the control group was significantly enhanced compared to the Ad.EGFP-MSC and Ad.SDF-1-MSC groups (Figs. 6A and 6B). Western blot also showed that HGF protein level in the Ad.EGFP-MSC and Ad.SDF-1-MSC groups increased more than that in the control group (Figs. 6D and 6E). More importantly, these changes were more obvious in

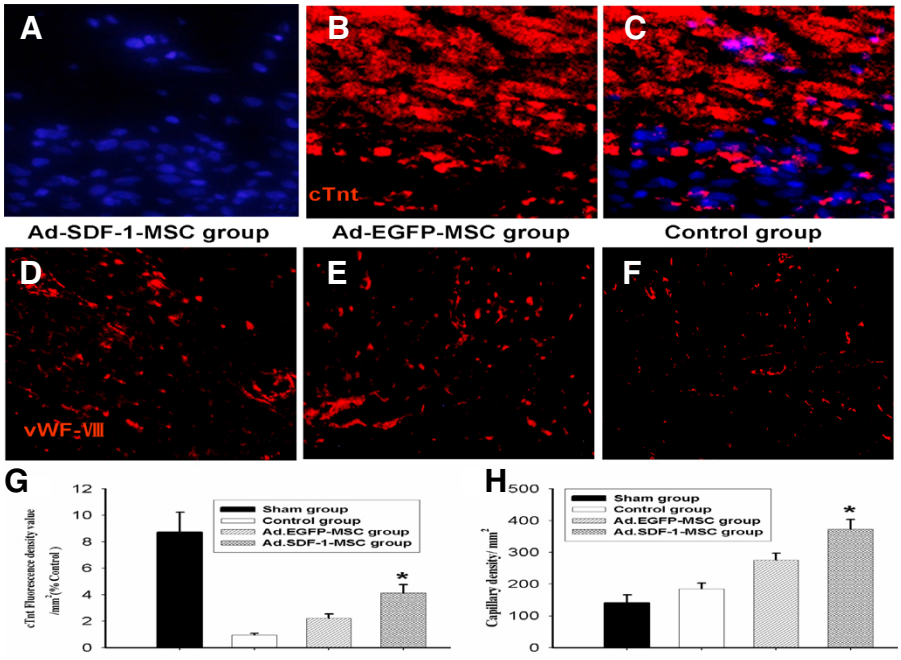

Fig. 4. Effects of transplantation of MSCs transfected with Ad-SDF-1 on angiogenesis. (A-C) Expression of cTnt in MSCs in the infarcted area after transplantation. (A) DAPI-labeled MSCs show blue fluorescence. (B) cTnt show red fluorescence. (C) Merging of DAPI-labeled MSCs and cTnt staining (white arrow) (400×). (D-F) Photomicrography shows representative immunohistochemical vwVIII factor staining of infarcted myocardium harvested on day-28. (E) Semi-quantitative assay of capillary density. n = 6 per group and **P* < 0.001 vs. the other groups.

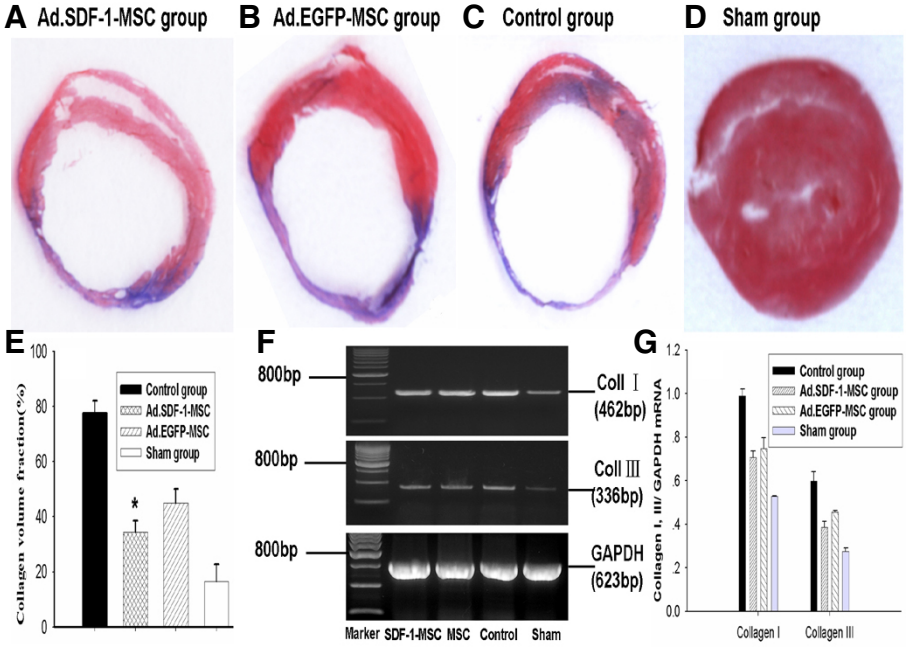

Fig. 5. (A-D) Representative myocardial sections showed markedly decreased cardiac fibrosis after Ad-SDF-1-MSC transplantation. (E) Quantitative analysis of collagen fraction showing that Ad-SDF-1-MSC transplantation significantly decreased cardiac fibrosis. n = 6. (F) Semi-quantitative RT-PCR analysis of mRNA levels. Expression levels of collagen I (col1) and collagen III (col3) were significantly decreased in the Ad-SDF-1-MSC and Ad-EGFP-MSC groups compared with control group. n = 6, **P* < 0.05 vs. the other groups, ***P* < 0.05 vs. the other groups.

the Ad.SDF-1-MSC group than the Ad.EGFP-MSC group.

Effects of SDF-1 modified MSCs transplantation on heart structure

Marked increases in the average left ventricle-wall thickness were observed in the Ad.SDF-1-MSC hearts (Fig. 7C). Moreover, infarct size was significantly reduced in Ad.SDF-1-MSC group compared to the control group (Fig. 7A). Quantitative analysis of cavitary dilatation and wall thinning in all hearts of three groups revealed that Ad.SDF-1-MSC injection animals displayed significantly less ventricular dilation than the control or Ad.EGFP-MSC groups. The expansion index was significantly lower in the Ad.SDF-1-MSC group than in the other groups (Fig. 7B).

Effects of SDF-1 modified MSCs transplantation on cardiac function

To confirm whether the overexpressing-SDF-1 MSCs in the injured myocardium would further improve cardiac function, we measured hemodynamics 28 days after transplantation and found that left ventricle function was significantly lower in the Ad.SDF-1-MSC, Ad.EGFP-MSC, and control groups compared to the sham group. However, improvement of left ventricle function was significantly greater in the Ad.SDF-1-MSC group compared to the other groups. The differences of LVSP ($P < 0.05$),

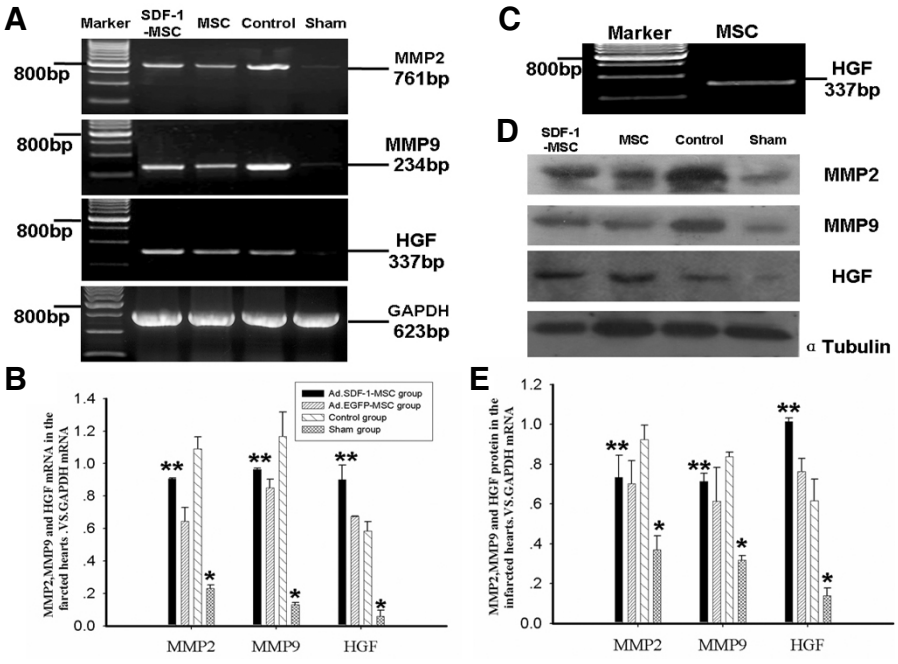

Fig. 6. (A) RT-PCR analysis of mRNA levels. Expression of MMP-2 and MMP-9 was significantly decreased and expression of HGF was obviously increased in Ad-SDF-1-MSC group compared to control group. (B) Semi-quantitative assay of MMP-2, MMP-9, and HGF mRNA expressions by optical density value. (C) RT-PCR analysis of HGF expression in MSCs. (D) Western blotting analysis of MMP-2, MMP-9, and HGF protein levels. (E) Semi-quantitative assay of MMP-2, MMP-9, and HGF protein expression by optical density value. n = 6, $^{*}P < 0.05$ vs. other groups, $^{**}P < 0.05$ vs. control and Ad-EGFP-MSC groups.

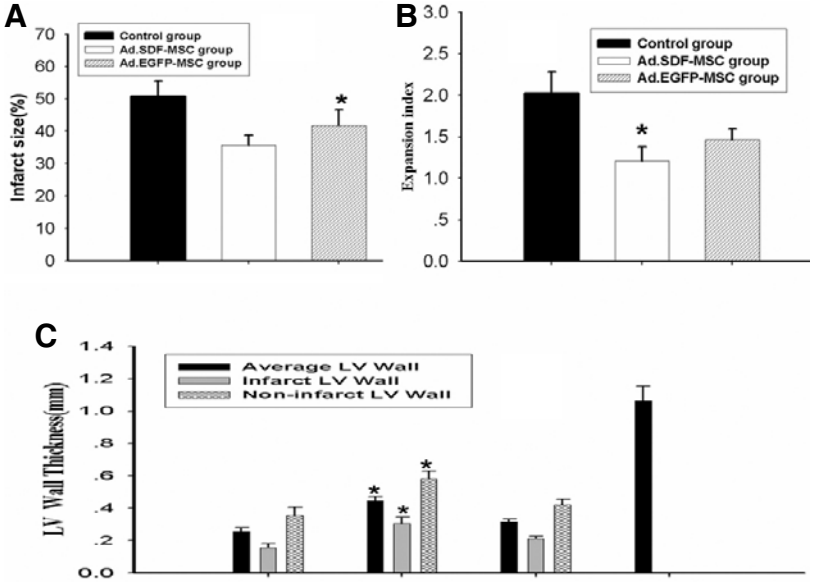

Fig. 7. (A) There were statistically significant differences in infarct size among the control, Ad-EGFP-MSC, and Ad-SDF-1-MSC groups. (B) Expansion index analysis in the Ad-SDF-1-MSC group was lower than in the control or Ad-EGFP-MSC groups. (C) Effects of Ad-SDF-1-MSC on LV-all thickness. The average LV wall in Ad-SDF-1-MSC group was thicker than in control or Ad-EGFP-MSC groups. n = 6, $^{*}P < 0.001$ vs. the other groups.

$\pm$ dp/dt_{max} and LVEDP ($P < 0.05$) were statistically significantly different between the three groups (Fig. 8).

DISCUSSION

In this study, we focused on the effects of overexpressing-SDF-1 MSCs on ventricle remodeling in a model of myocardial infarction of rat and found that they (1) enhanced MSC survival and paracrine function in the transplanted area, (2) promoted angiogenesis in the infarcted hearts, and (3) transcriptionally inhibited type I and III collagen, MMP-2, and MMP-9 gene expression.

It has been showed recently that MSC therapy increases regional perfusion by two mechanisms: direct effects (angiogenesis and vasculogenesis induced by implanted MSCs) and paracrine effects (angiogenic factors or arteriogenic cytokines secreted by implanted MSCs) (Pittenger et al., 1999; Schuleri et al., 2007). Both parameters might play equally important roles in neovascularization. However, the low survival rate of transplanted MSCs in infarcted hearts has limited their therapeutic effects. It is therefore important to develop a novel, efficient pro-survival (eg: Bcl2, Akt, et al) and angiogenic gene transfer into MSCs (Gnecchi et al., 2006; Li et al., 2008; Liu et al., 2008; Pons et al., 2008). In the present study, we demonstrated that MSCs could be transfected with recombinant adenovirus carrying hSDF-1 with high efficiency and without any adverse effect on cell viability. hSDF-1-modified MSCs expressed hSDF-1 at both mRNA and protein levels, and hSDF-1 secretion was detectable for as long as 21 days post-transfection. More impor-

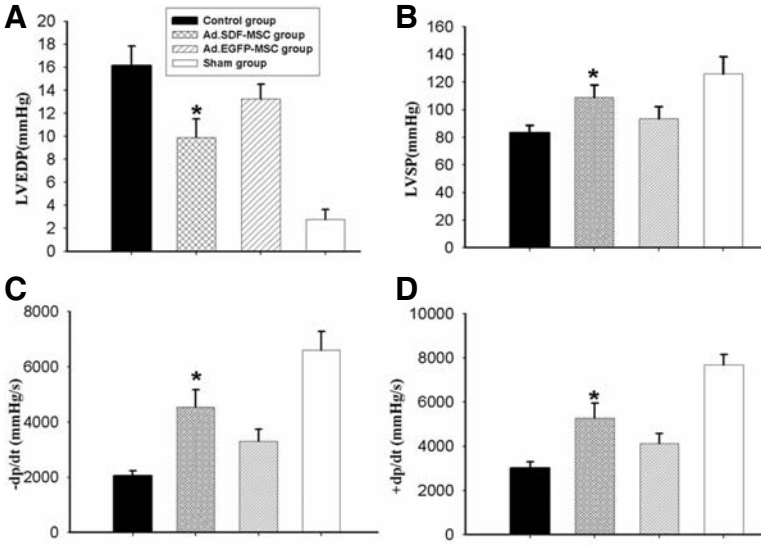

Fig. 8. Effects of Ad-SDF-1-MSC transplantation on hemodynamic parameters. LV function under baseline resting conditions 4 weeks after treatmen. LVSP: left ventricle systolic pressure; LVEDP: left ventricle end-diastolic pressure; + dP/dt_{max} and - dP/dt_{max}: rate of rise and fall of ventricular pressure, respectively. There were statistically significant differences among the control, Ad-EGFP-MSC, and Ad-SDF-1-MSC groups. *$p < 0.001$ vs. the other groups, n = 10.

tantly, sustained hypoxia did not damage hSDF-1-modified MSCs. This result was consistent with those of several reports, which showed that preconditioning with SDF-1 or overexpressing-SDF-1 suppressed MSCs apoptosis and enhanced their survival and engraftment (Pasha et al., 2008; Zhang et al., 2007). Furthermore, after transplantation of these cells, we also found that hSDF-1-modified MSCs survived a long time and effectively expressed hSDF-1 protein at least for 4 weeks in the injected area. These results indicate that hSDF-1 transfection might help the survival of transplanted MSCs, which might also help sustainable release of hSDF-1.

Meanwhile, compared to the Ad.MSC-EGFP group, the Ad. SDF-1-MSC group showed a 32.7% enhancement of angiogenesis. It has been reported that MSCs can themselves secrete several angiogenic cytokines (e.g. VEGF and HGF) to stimulate angiogenesis (Kinnaird et al., 2004). The present study demonstrates that intra-myocardial transplantation of Ad. SDF-1-MSC enhanced myocardial angiogenesis, and this is consistent with the report of Pasha and colleagues (Pasha et al., 2008). We have also shown that hSDF-1 was expressed in the infarcted areas treated with Ad.SDF-1-MSC 1 week after cell transplantation. Therefore, transplanted cells could supply a transient high level of SDF-1 to infarcted hearts. SDF-1 is a major chemotactic factor for stem cells and is also a retention signal for angiocompetent stem cells (e.g. EPCs and cardiac stem cells) (Pasha et al., 2008; Schuh et al., 2007; Tang et al., 2008; 2009b). Additionally it recruits pericyte and smooth muscle cells to stabilize and mature newly formed blood vessels (Ruiz et al., 2006). It has been shown to enhance collateral vessel formation in an animal model with myocardial and hindlimb ischemia. Previous studies had shown that SDF-1 gene therapy could enhance myocardial angiogenesis in the presence of endogenous VEGF in the ischemic areas (Hiasa et al., 2004; Shao et al., 2008; Tang et al., 2009a). The expression of hSDF-1 might therefore provide angiogenic effects in ischemic myocardium.

Cardiac myocyte apoptosis can trigger compensatory myocardial remodeling leading to heart failure. In the present study, we found that the infarcted ventricular wall was thicker and the infarction area became smaller in the Ad.SDF-1-MSC group compared to the Ad.MSC-EGFP group, and that the expansion index of left ventricular was less in the Ad.SDF-1-MSC group. These results indicate that in addition to its angiogenic effects, SDF-1 may provide myocardial protection against ischemic injury. In our previous work and in other studies, SDF-1 was able to promote survival of cardiac myocytes via changes in the SDF-1/CXCR4 axis (Saxena et al., 2008; Tang et al., 2008; Zhang et al., 2007). Interestingly, in the present study, engrafted Ad-SDF-1-MSCs could also differentiate into cardiomyocytes in the ischemic myocardium, which is agreement with the report of Chen and colleagues (Chen et al., 2008). However, the result is inconsistent with the report of Zhang and colleagues (Zhang et al., 2007). We presumed that this difference is related to the time of cell transplantation after myocardial infarction (Li et al., 2001). The expression of hSDF-1 might therefore provide myocardial regeneration and cardioprotective effects in ischemic myocardium. Hemodynamics measurements further suggested that Ad.SDF-1-MSC transplantation significantly improved left ventricle systolic and diastolic function. These results indicate that improved blood supply through angiogenesis would both preserve favorable geometry and inhibit collagen deposition, consequently attenuating remodeling and improving heart function.

Ventricular remodeling may be involved in both systolic and diastolic dysfunction of the failing heart. In the failing heart, normal collagens are degraded by increased levels of MMPs, and fibrous interstitial deposits of poorly cross-linked collagens are synthesized. This, in turn, may lead to myocardial fibrosis, dilation of the ventricles, and cardiac dysfunction. Preserving collagen homeostasis is likely to ameliorate heart failure. Matrix metalloproteinases (MMPs) play an important role in ventricular remodeling by degrading extracellular matrix (ECM). Inhibition of MMP activities prevented progressive left ventricle remodeling and improved heart function in an animal model of heart failure (Ohnishi et al., 2007). Some studies showed MSC transplantation reduced left ventricle remodeling through decreased expression of MMP2, MMP9, collagen type I and type III, and transforming growth factor- beta1(TGF-β1) in the infarcted hearts (Liu et al., 2008; Mangi et al., 2003; Novotny et al., 2008; Sun et al., 2007; Xu et al., 2005). Furthermore, Ohnishi recently reported that the anti-fibrotic effect of MSCs was related to its paracrine actions, which attenuated cardiac fibroblast proliferation and collagen synthesis (Ohnishi et al., 2007). In our study, we found that the expression of collagen type I and type III clearly decreased in the Ad.SDF-1-MSC and Ad.MSC-EGFP groups. The expression of MMP-2 and MMP-9 increased in the

infarcted areas of the Ad.SDF-1-MSC group compared to that that of sham and Ad.MSC-EGFP groups, but it was yet a significant slightly decrease in Ad.SDF-1-MSC group than control group. These changes could contribute to the degradation of collagen in the infarcted areas, and inhibit the formation of poorly cross-linked collagens, which could result in attenuation of cardiac fibrosis and improvement of heart function.

In addition, interestingly, stem/progenitor cell itself expresses matrix metalloproteinases (MMPs), and SDF-1 could stimulate the secretion of MMP-2, MMP-9, and other metalloproteinases in these cells (Barkho et al., 2008; Higashiyama et al., 2007; Janowska-Wieczorek et al., 2000; Kollet et al., 2003; Shao et al., 2008; Son et al., 2006). Furthermore, overexpression of SDF-1 mediated by adenoviral vectors in the infarcted hearts involved MMPs up-regulation, which contributed to create a favorable tissue environment for stem cell migration into the injured hearts (Elmadbouh et al., 2007; Tang et al., 2009b). Collagen degradation is mediated by MMP-1, -2, and -9, which are known to be elevated during the necrotic phase of infarct healing, involved in disruption of the collagen network, and associated with increased CXCR4 expression on stem cells. HGF accelerates recruitment of stem cell into fibrotic liver by stimulating the SDF-1/CXCR4 axis and suppresses the progression of liver fibrosis (Higashiyama et al., 2007; Inagaki et al., 2007; Kollet et al., 2003). Here, overexpressing-SDF-1-MSCs transplantation could improve cardiac function at least in part through an anti-fibrotic effect in a rat model of myocardium infarction, and this anti-fibrotic effect was related to the increased expression of MMP-2 and MMP-9 in the infarcted hearts. Therefore, overexpressing-SDF-1 MSCs could prevent heart fibrosis and remodeling through their paracrine function in infarcted hearts.

HGF is a growth factor that has been shown to exert antifibrotic, antiapoptotic, and angiogenic effects (Aoki et al., 2000; Miyagawa et al., 2006; Nakamura et al., 2000; Taniyama et al., 2002; Ueda et al., 2001; Yang et al., 2006). It has been reported that MSCs can themselves secrete several angiogenic cytokines (e.g. VEGF and HGF) to stimulate angiogenesis (Kinnaird et al., 2004). Overexpressing-Akt MSCs could enhance the production of secreted factors (e.g. HGF) in MSCs (Gnecchi et al., 2006; Mangi et al., 2003). Recently, preconditioning with SDF-1 induced Akt phosphorylation and reduced apoptosis in MSCs and increased the expression of VEGF and angiogenesis mediated by VEGF in the infarcted hearts. Furthermore, the improved blood supply through angiogenesis would inhibit collagen deposition, consequently attenuating remodeling and improving heart function. (Pasha et al., 2008). In the present study, the overexpressing-SDF-1 MSCs also activated the prosurvival gene Akt, and the increased production of HGF. Interestingly, previous studies have shown that HGF levels in rat infarcted myocardium were significantly increased compared with those in normal hearts, and this was attributable to repression of angiotensin II and TGF-β1 (Nakamura et al., 2002; Taniyama et al., 2002; Ueda et al., 2001). Transfection of an HGF expression vector into the myocardium of these rats significantly decreased the size of the fibrotic area (Aoki et al., 2000; Miyagawa et al., 2006; Wang et al., 2004; Yang et al., 2006). Consistent with previous studies, our study also showed higher levels of HGF expression in the LAD ligation-induced failing hearts than in the sham-treated hearts. More importantly, we also showed *in vivo* that transplantation of Ad.SDF-1-MSCs or Ad.EGFP-MSCs increased the expression both of HGF mRNA and HGF protein in the myocardium; furthermore, the effects was significantly enhanced in the Ad.SDF-1-MSCs group and this was related to the increased survival of MSCs mediated by SDF-1. These observations therefore suggest that HGF derived from overexpressing-SDF-1-MSCs- may participate in the amelioration of myocardial fibrosis. MSCs can secrete several cytokines, but further studies will be required to detect other cytokines which may also mediate the antifibrotic effect, and determine the central role of HGF in the antifibrotic effect compared with released other cytokines of MSCs.

CONCLUSION

SDF-1-modified MSCs enhanced the tolerance of engrafted MSCs to hypoxic injury *in vitro* and improved their viability in infarcted hearts, thus helping preserve the left ventricle contractile function and attenuate left ventricle remodeling. This may be at least partly mediated by enhanced paracrine signaling from MSCs via antifibrotic factors such as HGF.

ACKNOWLEDGMENTS

We thank Professor Yongsheng Ren for his helpful instruction in heart function evaluation. We are grateful to Long Chen for help with immunofluorescence staining. This study was supported by grants from National Natural Science Foundation of China (30700306), Hubei Natural Science Foundation (2005 ABA079), Hubei Health Department Science Foundation (JX3B29), Hubei Education Department Science Foundation (Q200524003, B200624006), YunYang Medical College Science Foundation (2005QDJ01), and Shiyan Renmin Hospital Science Foundation, China.

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