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PRECLINICAL RESEARCH

Improved Graft Mesenchymal Stem Cell Survival in Ischemic Heart With a Hypoxia-Regulated Heme Oxygenase-1 Vector

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OBJECTIVES The goal of this study was to modify mesenchymal stem cells (MSCs) cells with a hypoxia-regulated heme oxygenase-1 (HO-1) plasmid to enhance the survival of MSCs in acute myocardial infarction (MI) heart.

BACKGROUND Although stem cells are being tested clinically for cardiac repair, graft cells die in the ischemic heart because of the effects of hypoxia/reoxygenation, inflammatory cytokines, and proapoptotic factors. Heme oxygenase-1 is a key component in inhibiting most of these factors.

METHODS Mesenchymal stem cells from bone marrow were transfected with either HO-1 or LacZ plasmids. Cell apoptosis was assayed in vitro after hypoxia-reoxygen treatment. In vivo, 1×10^6 of male MSC_{HO-1}, MSC_{LacZ}, MSCs, or medium was injected into mouse hearts 1 h after MI (n = 16/group). Cell survival was assessed in a gender-mismatched transplantation model. Apoptosis, left ventricular remodeling, and cardiac function were tested in a gender-matched model.

RESULTS In the ischemic myocardium, the MSC_{HO-1} group had greater expression of HO-1 and a 2-fold reduction in the number of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate in situ nick end labeling-positive cells compared with the MSC_{LacZ} group. At seven days after implantation, the survival MSC_{HO-1} was five-fold greater than the MSC_{LacZ} group; MSC_{HO-1} also attenuated left ventricular remodeling and enhanced the functional recovery of infarcted hearts two weeks after MI.

CONCLUSIONS A hypoxia-regulated HO-1 vector modification of MSCs enhances the tolerance of engrafted MSCs to hypoxia-reoxygen injury in vitro and improves their viability in ischemic hearts. This demonstration is the first showing that a physiologically inducible vector expressing of HO-1 genes improves the survival of stem cells in myocardial ischemia. (J Am Coll Cardiol 2005;46:1339–50) © 2005 by the American College of Cardiology Foundation

A leading cause of heart failure is myocardial ischemia, which precipitates dysfunction and the death of cardiomyocytes (1). Adult stem cells have been proposed as a promising source for the repair and regeneration of heart cells and for the restoration

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of heart function (2–4). However, progress in stem cell therapy is hampered by the poor survival of implanted cells. A high level of engrafted cell death occurs within four days after grafting into injured hearts (5,6). Furthermore, regenerated tissue from stem cells does not survive repeated bouts of ischemia (7). The molecular mechanism for stem cell death in ischemic heart is in large part because of ischemia; moreover, endogenous and environmental factors, such as ischemia-reperfusion, inflammatory response, and proapoptotic factors, play important roles. Therefore, cytoprotection for one week is critical for improving the efficiency of cell therapy.

We have developed a hypoxia-inducible vector system that can induce transgene expression in response to hypoxia in the heart without the need for exogenous stimuli, such as drugs (8–11). Heme oxygenase-1 (HO-1), an antiapoptotic and antioxidant enzyme, possesses potent cytoprotective activity in an ischemic environment (12,13). To develop a strategy aimed at enhancing stem cell viability in the short-term and at enhancing stem cell resistance to ischemia, we transfected mesenchymal stem cells (MSCs) with the hypoxia-regulated system and hypothesized that HO-1 could be switched automatically on by hypoxia and confer timely cellular protection to MSCs in danger. We proposed that gene modification of stem cells with hypoxia-inducible HO-1 vector would improve stem cell viability and thereby potentially improve stem cell therapy for heart disease.

METHODS

Generation of hypoxia-inducible human HO-1 plasmid. The hypoxia-inducible plasmid system is a double-plasmid system (Fig. 1) containing a sensor plasmid with the

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Abbreviations and Acronyms

cTNT	= cardiac troponin T
DAPI	= 4′,6-diamidino-2′-phenylindole
FITC	= fluorescein isothiocyanate
HO-1	= heme oxygenase-1
IL	= interleukin
LV	= left ventricular
MI	= myocardial infarction
MSC	= mesenchymal stem cell
OST	= oxygen-sensitive toggle
PCR	= polymerase chain reaction
TRITC	= tetramethyl rhodamine isothiocyanate
TUNEL	= terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate in situ nick end labeling

oxygen-sensitive toggle (OST) and an effector plasmid with the cardiac protective gene (8–10). The sensor plasmid (pS-OST) contains an oxygen-dependent degradation domain (amino acids 394 to 603). This domain was amplified by polymerase chain reaction (PCR) from pCEP4/HIF-1-alpha and inserted in the frame between the coding sequence of GAL4DNA binding domain and p65 activation domain in pS-CMV to generate pS-OST (Fig. 1). The effector plasmid (pE-hHO-1) was constructed by replacing the LacZ coding sequence with full-length cDNA for human heme oxygenase-1 (hHO-1) in pE/V5-His/LacZ (Invitrogen Corporation, Carlsbad, California). A six-copy His tag was added to the C-terminal of hHO-1. Human HO-1 is driven by six copies of a 17-bp GAL4 UAS and an adenovirus-derived E1b TATA box. The construction of the plasmids was confirmed by nucleotide sequence analysis.

Animals. All studies were performed with the approval of the University of South Florida Institutional Animal Care and Use Committee. The investigation conformed to the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. The syngenic male and female mouse strain BAL B/c (Harlan) weighing 20 to 25 g was used to avoid rejection.

Cell isolation, culture, transfection, and labeling. Bone marrow was flushed from tibias and femurs of male donor mice using a 25-gauge needle. Whole marrow cells were cultured at $1 \times 10^6/cm^2$ in MesenCult basal medium that was supplemented with MSC stimulatory supplements (StemCell Technologies Inc., Vancouver, BC, Canada). The nonadherent cells were removed by a medium change at 72 h and every four days thereafter. The monolayer, referred to as MSCs, was expanded by two passages.

A Polyethylenimine-Transferinfection Kit (Tf PEI-Kit; Bender MedSystems, Burlingame, California) was prepared according to commercial protocol. Briefly, two plasmids (1:1) were mixed with a Tf PEI to generate a Tf PEI/DNA complex suspension. Mesenchymal stem cells at 70% confluence were incubated with the Tf PEI/DNA containing the sensor plasmid and effector plasmid for 4 h at 37°C. Control cells were transfected with inducible vector with LacZ as reporter using the same protocol.

Before implantation, MSCs were trypsinized, washed, and labeled with 4′,6-diamidino-2′-phenylindole (DAPI) (Sigma, St. Louis, Missouri), as previously described (14). In brief, sterile DAPI stock solution was added to culture medium at a final concentration of 50 μg/ml for 30 min. After labeling, cells were washed six times in D-Hanks solution to remove excess unbound DAPI. The DAPI stains were 100% of the MSCs nuclei.

In vitro experiments. HYPOXIA PROTOCOL AND REPORTER GENE EXPRESSION. To evaluate hypoxia regulation of hypoxia-inducible hHO-1 vectors and hypoxia-inducible LacZ in vitro, cells were incubated on eight-well Lab-Tek Chamber Slides (Nalge Nunc International, Rochester, New York). Cell transfection and hypoxic treatment was performed as described previously (8). Mesenchymal stem cells were transfected with 1 μg of pS-OST and 0.5 μg of pE-hHO-1/pE-LacZ. Twenty-four hours after transfection, the medium was changed and MSCs were incubated at 1% or 20% O_2 for 24 h before the preparation of lysates. Each condition was run in triplicate.

LacZ expression was assayed using "LacZ Cell Staining Kit" according to the manufacturer's protocol (InvivoGen, San Diego, California). For immunofluorescent examination of hHO-1, samples were incubated in a 1:50 dilution of antihuman HO-1 antibody at 37°C for 30 min, followed by incubation in a 1:500 dilution of fluorescein isothiocyanate (FITC)-conjugated second antibody (Sigma).

Western blot analysis was performed using 10 to 15 μg of whole cell. The hHO-1-6xHis fusion protein was probed with monoclonal anti-6xHis antibody (Invitrogen). The internal control proteinglyseraldehyde-3-phosphate dehy-

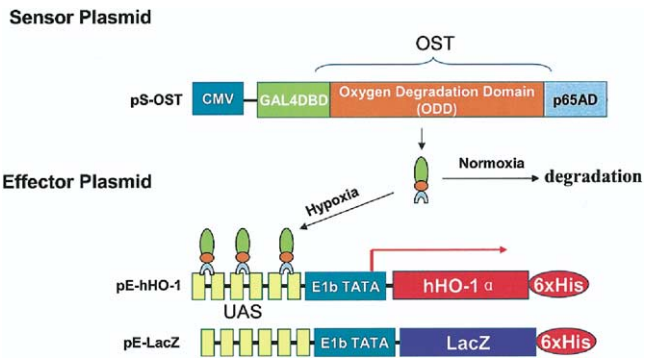

Figure 1. Diagram of hypoxia-regulated plasmid system, which can amplify the power of a promoter based on the strong transcription activity of the GAL4/p65 fusion protein. The ischemic biosensor is composed of an oxygen-sensing toggle (OST), which is a GAL4DNA-binding domain, ODD of the hypoxia-inducible factor-1-alpha along with p65 activation domain. The effector plasmid contains GAL4 upstream activation sequence (UAS) in front of an adenovirus E1b TATA box and the hHO-1-6xHis or LacZ fusion gene. Hypoxia inducible LacZ system is replaced hHO-1 gene with LacZ.

drogenase (GAPDH) antibody (Chemicon, Temicula, California) was probed. The antigen-antibody complexes were visualized by enhanced chemiluminescence (Amershan plc, Buckinghamshire, United Kingdom).

HYPOXIA-REOXYGEN PROTOCOL. To test the capability of resistance to ischemia/reperfusion damage of MSCs, we used hypoxia/reoxygen/hypoxia treatment on MSC_{HO-1}, MSC_{LacZ}, and MSCs with 24 h of hypoxia (1% O_2), 1 h of reoxygen (20% O_2), followed by another 24 h of hypoxia.

DETECTION OF APOPTOSIS CAUSED BY HYPOXIA/REOXYGEN INJURY. For detection of the proapoptosis protein Bax, monoclonal anti-Bax antibody (Upstate Biotechnology, Charlottesville, Virginia) was used in Western blot. For immunofluorescent analysis, MSCs were seeded into chamber slides at day 2 after transfection for hypoxia-reoxygen treatment. After that, samples were fixed with 4% formaldehyde. Half samples were incubated in a 1:50 dilution of antihuman HO-1 antibody at 37°C for 30 min, followed by incubation in a 1:500 dilution of tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (Sigma). For the other samples, we used the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate in situ nick end labeling (TUNEL) assay according to manufacturer's protocol (Upstate). Nuclei were counterstained with DAPI. The degree of apoptosis is calculated as the number of TUNEL cells per 500 nuclear numbers of MSCs.

In vivo experiments. MYOCARDIAL INFARCTION (MI) MODEL AND CELL IMPLANTATION. Male BAL B/c mice were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and mechanically ventilated. The sham operation was made by passing suture around the left coronary artery without ligation. After the heart was exposed through a lateral thoracotomy, an 8-0 polypropylene thread was passed around the left coronary artery and the artery was occluded. At day 3 after gene transfection, MSCs were harvested using trypsin and resuspended in serum-free Dulbecco's modified Eagle's medium just before grafting to the heart. The male MSCs were used for the following three-part experiments: 1) Gender-mismatched transplants for quantifying survival MSCs by real-time PCR: 1.2×10^6 of hHO-1- transfected-male MSCs (MSC_{HO-1}) and LacZ-transfected male MSCs (MSC_{LacZ} group) in 50 μl of volume were injected into syngenic female BAL B/c mouse hearts 1 h after MI with a 30-gauge needle (n = 16/group). 2) Gender-matched transplants for remodeling and functional comparison: 1×10^6 of MSC_{HO-1}, MSC_{LacZ}, MSCs, or medium (n = 16/group) in 50 μl of volume were injected into male BAL B/c mouse hearts 1 h after MI. 3) To test the role of HO-1 in preventing inflammation, a group of MI mice were injected intramyocardially with hypoxia-regulated hHO-1 or LacZ plasmid (n = 10/group) 1 h after MI as described previously (15).

REAL-TIME PCR. Genomic DNA was isolated using Genomic-tip Kit (Qiagen, Amsterdam, the Netherlands). Real-time PCR was conducted as described elsewhere (16) on a 5700 sequence detection system (Applied Biosystems Group, Foster City, California). Standard curves were generated by serially diluting murine male genomic DNA prepared from male MSCs. The sequence of the PCR primers and probe used for detection of murine male-specific Sry gene were as follows (17):

Forward primer: 5′-GGAGAGAGGCACAAGTTGGC-3′;
Reverse primer: 5′-TTCCAGCTGCTTGCTGATC-3′
Taqman probe: 5′-FAM-CAACAGAATCCCAGCATG CAGAATTCAG-TAMRA-3′

The measurement of Sry genes in MSC_{HO-1}- and MSC_{LacZ}-grafted hearts was conducted at 4 h (n = 3/group), 24 h (n = 3/group), and 7 days (n = 3/group) after gender-mismatch transplants.

ASSAY FOR THE INFLAMMATORY RESPONSE IN PLASMID-TREATED HEARTS. Western blot analysis was performed using 125 μg of protein from HO-1- and LacZ plasmid-treated hearts one day after injection (n = 3/group). The interleukin (IL)-1β protein was probed with monoclonal anti–IL-1β antibody (Invitrogen). The internal control protein GAPDH antibody was probed. The ratio of IL-1β: GAPDH was compared between two group using Quantity One software (Bio-Rad, Hercules, California). Immunostaining for CD45 was performed four days after plasmid injection, and the tissue sections were fixed in 4% formaldehyde for 10 min. Sections of HO-1- and LacZ plasmid-treated hearts were incubated with a 1:50 dilution of biotin-conjugated rat anti-mouse CD45 antibody (BD Bioscience, San Jose, California) using DakoCytomation The ARK (Animal Research Kit) (DakoCytomation, Carpinteria, California).

ASSAY FOR REPORTER GENE EXPRESSION IN GRAFTED CELLS AT ISCHEMIA. Three hearts from MSC_{HO-1}- and MSC_{LacZ}-engrafted hearts were embedded in Tissue-Tek OCT (Sakura Finetek U.S.A. Inc., Torrence, California) at four days after implantation, 5-μm cryostat sections were obtained in MSC_{HO-1} tissue, whereas three adjacent sections with 5-, 20-, and 5-μm sequence were cut in MSC_{LacZ}. The sections of MSC_{HO-1} and MSC_{LacZ} were incubated with a 1:50 dilution of antihuman HO-1 antibody followed by incubation in a 1:500 dilution of TRITC-conjugated secondary antibody. To identify LacZ expression in ischemic myocardium, a 20-μm section of MSC_{LacZ} heart was stained using the LacZ Tissue Staining Kit according to manufacturer's protocol (InvivoGen).

FUNCTIONAL ASSESSMENT. At 14 days after cell transplantation, mice from gender-match transplant groups and sham group (n = 6 in each group) were performed invasive left ventricular (LV) hemodynamic measurement in closed-chest preparation as described (18). Mice were

anesthetized by pentobarbital (40 mg/kg, i.p.). Mikro-Tip pressure catheter transducers (Model SPR-671, Millar Instruments Inc., Houston, Texas) were cannulated into the LV chamber through right carotid artery. The LV pressure was digitized using the commercially available data acquisition system (PowerLab/8sp, ADinstruments Inc., Mountain View, California). After steady state had been established, maximal rates of pressure increase and decrease (±dP/dt) were recorded in the closed-chest preparation.

REMODELING, APOPTOSIS, AND CELL DIFFERENTIATION. After invasive LV functional detection, mice were killed with an overdose of pentobarbital. Hearts at the papillary muscle level were selected and embedded in Tissue-Tek OCT (Sakura). Cryostat sections (5 μm each) were obtained in MSC_{HO-1}, MSC_{LacZ}, MSCs, and medium for Masson staining and immunofluorescent staining. Masson trichrome staining was performed using a commercial kit according to manufacturer's protocol (Sigma). Sections from all slices were projected onto a screen for computer-assisted planimetry. The percentage of fibrotic area in a total LV area and the thickness of infarct wall were assayed by analySIS (Soft Imaging System GmbH, Lakewood, Colorado). We performed the TUNEL assay according to the manufacturer's protocol. Sections were counterstained with Eriochrome Blue-Black R (LabChem, Pittsburgh, Pennsylvania). The percentage of apoptosis is calculated as the number of TUNEL-positive cell number/DAPI-labeled nuclear numbers of MSCs × 100. An average of 200 cells per slide was counted. The slices from MSC_{HO-1} also were used to assay the alpha-sarcomeric actinin, cardiac troponin T (cTnT), and CD31 expression. The sections were incubated with 1:50 dilution of monoclonal anti–alpha-sarcomeric actin (Sigma) and polyclonal anti-cTnT (Advanced Immunochemical, Long Beach, California) followed by incubation with a 1:500 dilution of TRITC-conjugated anti-rabbit antibody and FITC-conjugated antimouse antibody. A 1:50 dilution of FITC conjugated monoclonal anti-CD31 (Sigma) was used to identify endothelial cells.

Statistical analysis. All values are expressed as mean ± SEM. The Student *t* test was used for two-group comparison and analysis of variance followed by an unpaired Student *t* test with Bonferroni's correction was used for multiple group comparisons. Reported significances ($p < 0.05$, $p < 0.01$) were calculated by Bonferroni-adjusted pairwise comparisons between MSC_{HO-1} group with the MSC_{LacZ}-treated group, MSC group, or medium group for the respective parameter (Figs. 2B and 3C to 3F). Values of $p < 0.05$ were considered significant.

RESULTS

Human HO-1 expression protects against MSC apoptosis in vitro. A total of 80% of LacZ-transfected MSCs stained positively for beta-Gal after hypoxia treatment (1% O_2 for 24 h). Immunofluorescent staining demonstrated stronger hHO-1 expression in hypoxia-treated MSC_{HO-1} compared with MSC_{HO-1} in normoxia (Figs. 4A1 and 4A2). Beta-gal staining in vitro showed that there was a higher level expression of LacZ in MSC_{LacZ} during hypoxia compared with normoxia (Figs. 4A3 and 4A4). Western blotting showed that total hHO-1 levels in hypoxia-treated MSC_{HO-1} was on average 5.18-fold more abundant than at normoxia ($p < 0.001$) (Figs. 4B and 4C). Because hHO-1 was fused with 6xHis tag in the vector, the expressed fusion protein reacts only with antibody to 6xHis and does not react with endogenous mouse HO-1.

To test the capability of MSC_{HO-1} to resist ischemia/reperfusion damage, we applied hypoxia/reoxygen/hypoxia injury to MSC_{HO-1} with 24 h of hypoxia (1% O_2), 1 h of reoxygen (20% O_2), followed by another 24 h of hypoxia. Most of MSC_{HO-1} expressed HO-1 fusion protein in immunostaining whereas HO-1 expression was low in MSC_{LacZ} and MSCs. The increase in HO-1 expression in MSC_{HO-1} was accompanied by a decrease in the MSC apoptosis. As a result, the rate of cell apoptosis by TUNEL in MSC_{LacZ} or MSCs (5.06 ± 0.43 $TUNEL^+$ and 5.32 ± 0.46 $TUNEL^+$ per 500 cells, respectively) exceeded that of MSC_{HO-1} (3.00 ± 0.13 $TUNEL^+$ per 500 cells) by 1.7-fold ($p < 0.01$ for both MSC_{LacZ} and MSCs) (Fig. 5A). Similarly, a down-regulation in a proapoptotic gene Bax level in the cell lysate of MSC_{HO-1} was confirmed by Western blot in comparison with MSC_{LacZ} and MSCs (Fig. 5B).

Hypoxia-regulated hHO-1 plasmid administration decreases inflammatory cytokine levels and leukocyte infiltration. After plasmid intramyocardial injection in ischemic heart, we found a relative decrease in proinflammatory cytokines lL-1-beta in ischemic myocardium from the hHO-1–treated hearts compared with the LacZ-treated controls (OD ratio IL-1b/glyseraldehyde-3-phosphate dehydrogenase: 0.195 ± 0.046 vs. 0.916 ± 0.246, $p = 0.045$, $n = 3$ hearts/group) (Figs. 6A and 6B). Concomitant with the reduction in inflammatory factor, CD45-positive leukocyte infiltration was less in the HO-1 plasmid-treated hearts compared with the LacZ-treated hearts in the peri-infarct myocardium (Figs. 6C and 6D), indicating that the hHO-1 gene transfer inhibits inflammatory cell infiltration in ischemic heart.

Induction of human HO-1 expression in ischemic myocardium protects against MSC apoptosis in vivo. At 14 days after cell injection, the implanted cells were assessed in ischemic myocardium by TUNEL assay. In the MSC_{HO-1} group, we observed that a significantly smaller percentage of the implanted cells were TUNEL positive (2.32 ± 0.39 $TUNEL^+$ per 200) compared with the MSC_{LacZ} (4.98 ± 0.40 $TUNEL^+$ per 200, $p < 0.01$ vs. MSC_{HO-1}, $n = 6$) and MSCs group (5.54 ± 0.43 $TUNEL^+$, $p < 0.01$ vs. MSC_{HO-1}, $n = 6$) (Figs. 2A and 2B). This finding is inversely correlated with staining four days after plasmid implantation, which demonstrated that there was a higher level of hHO-1 expression in most of implanted MSC_{HO-1}

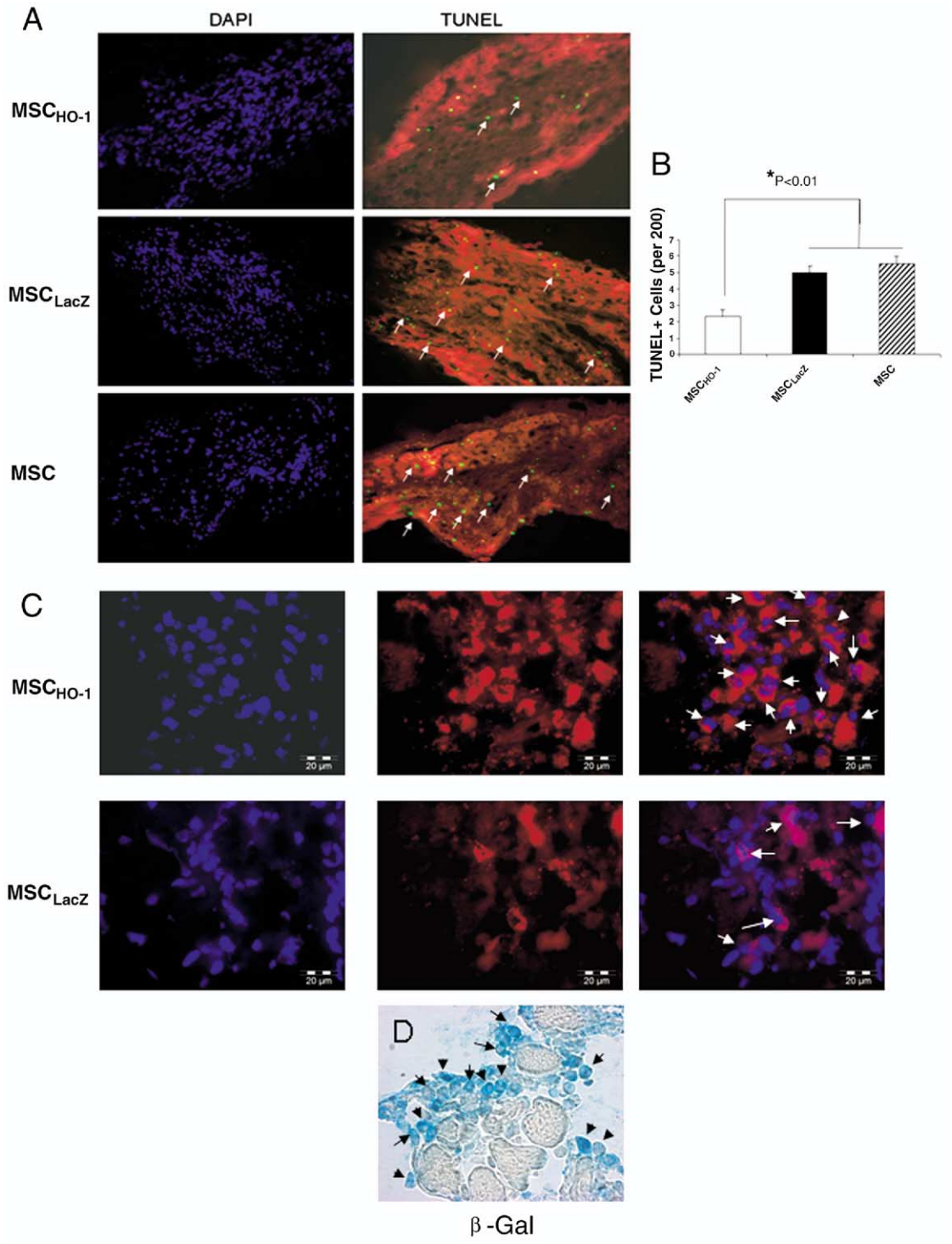

Figure 2. Effect of treating mesenchymal stem cells (MSCs) with hypoxia-inducible hHO-1 vector on grafted cell survival in ischemic myocardium. **(A and B)** Quantification of intramyocardial terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate in situ nick end labeling-positive graft cells two weeks after MSC injection. TUNEL-positive implanted cells were significantly less in MSC_{HO-1} group compared with the MSC_{LacZ} and MSCs group ($p < 0.01$, $n = 6$/group). **(C)** Immunofluorescent staining of hHO-1 to detect the expression of hypoxia-regulated gene in ischemic myocardium four days after implantation. The number of HO-1-positive graft cell was higher in the MSC_{HO-1} group than the MSC_{LacZ} groups. **(D)** At the peri-infarct area, donor MSC_{LacZ} stained positively for LacZ marker gene, indicating the ischemia-activated LacZ gene in MSC_{LacZ}. **Arrows** indicate the β-gal nuclei.

cells compared with the MSC_{LacZ} (Fig. 2C). Moreover, donor MSC_{LacZ} was stained positively for LacZ, indicating ischemia-activated LacZ gene in MSC_{LacZ} (Fig. 2D). The hypoxia-regulated HO-1 transfected MSCs did not lose their differentiation capacity in vivo. Some MSC_{HO-1} stained positively for alpha-sarcomeric actin and cTnT at peri-infarct area at 14 days after the transplantation (Figs. 7A to 7D). Some labeled MSC_{HO-1} stained positively for

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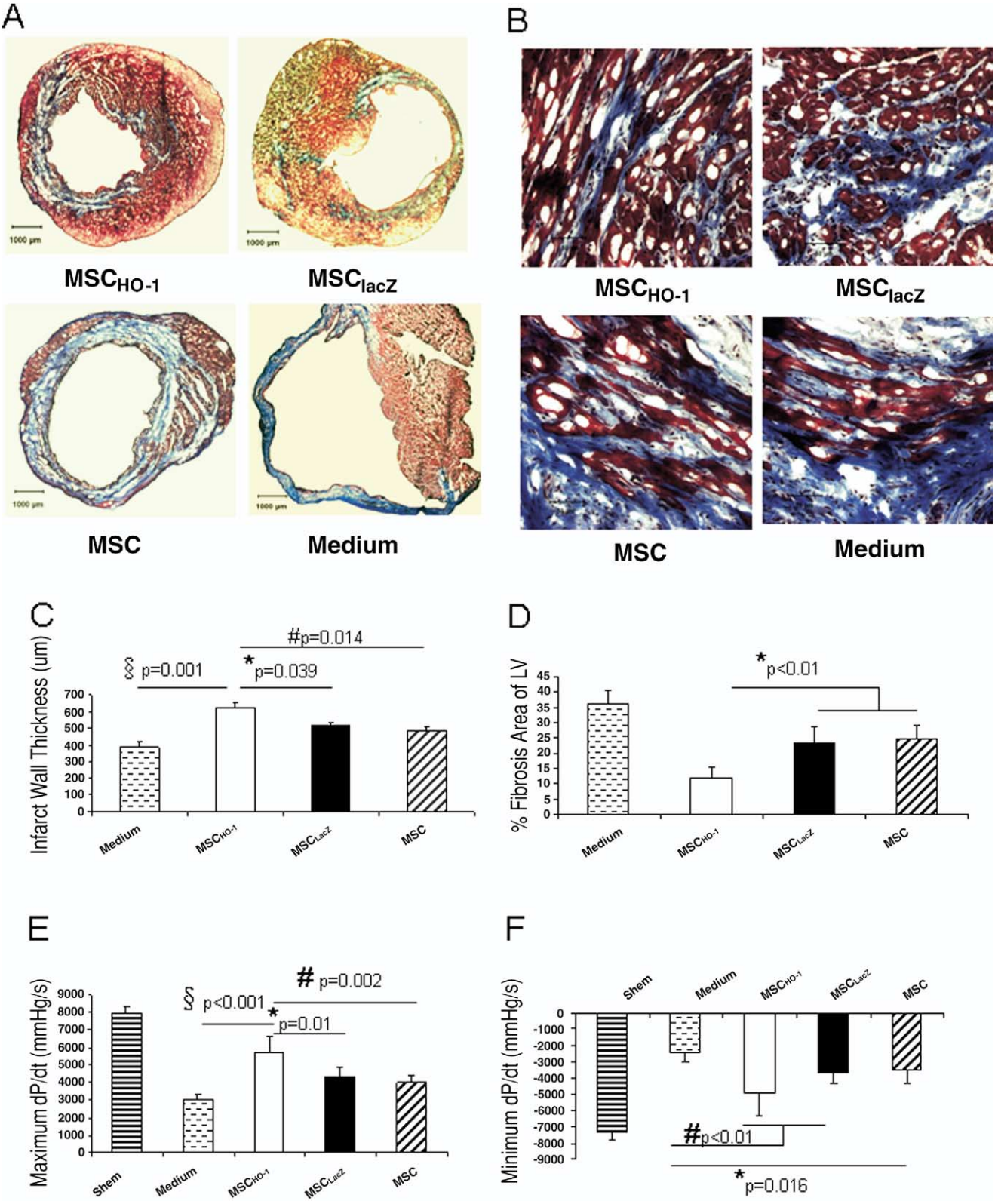

Figure 3. Injection of mesenchymal stem cells (MSCs) inhibits left ventricular (LV) remodeling and improves LV function. Masson trichrome staining showed infarct wall was significantly thicker in MSC_{HO-1} group in compared with other control groups **(A and C)**. The percent of fibrotic area in total LV area was significantly reduced in MSC_{HO-1} group compared with other control groups **(B and D)**. Left ventricular systolic performance and diastolic performance, as assessed by maximum dP/dt and minimum dP/dt, were best in the MSC_{HO-1} group, indicating that survival MSC_{HO-1} contribute to preserve systolic and diastolic functions of infarct heart. Moreover, the MSC_{LacZ} and MSCs demonstrated less LV remodeling and better hemodynamic function in comparison with the medium group **(E and F)**.

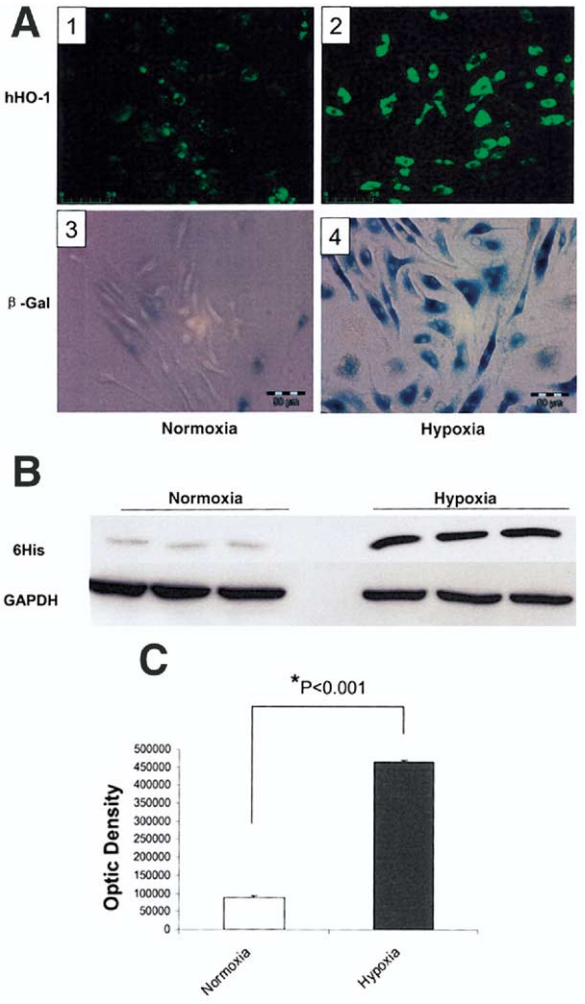

Figure 4. Hypoxia-induced overexpression of reporter gene in MSC_{HO-1} and MSC_{LacZ}. **(A)** Immunostaining for human HO-1 shows that 24 h of hypoxia increased the expression of hHO-1 expression compared with normoxia **(1 and 2)**. Beta-gal staining in vitro demonstrated that higher level expression of LacZ in hypoxia treated MSC_{LacZ} compared with normoxia **(3 and 4)**. Almost 80% transfected MSCs were positively stained for LacZ after hypoxic treatment. **(B)** Western blot demonstrated that the abundances of hHO-1 fusion protein were lower in MSC_{HO-1} at normoxia in comparison with hypoxia with 6xHis antibody for detection. **(C)** The hHO-1 fusion protein level of MSC_{HO-1} in hypoxia was 5.18-fold more abundant than in normoxia (mean ± SEM; $p < 0.001$; $n = 3$).

CD31 and formed microvessels (Figs. 7E to 7G), indicating that survival MSC_{HO-1} differentiated into cardiomyocytes and endothelial cells within the native myocardium.

Hypoxia-regulated HO-1 plasmid treatment enhances MSC survival in ischemic myocardium. We quantified engraftment levels of male, donor-derived cells in the ischemic hearts of female transplanted recipients using real-time PCR. Threshold cycle of the Sry gene in male MSCs have good relation with serially diluted cells (Figs. 8A and 8B), $R^2 = 0.9879$. Real-time PCR assay of Sry gene quantified the number of survival male MSCs in female hearts treated with MSC_{HO-1} and MSC_{LacZ} cells. The survival ratio = survival MSCs number/ total implanted cells. Survival ratio was assayed 4 h, 24 h, and 7 days after transplant, as shown in Figure 8C. The engrafted MSC survival was significantly higher in the MSC_{HO-1} than the MSC_{LacZ} group (4 h: 68.7 ± 6.2% vs. 21.5 ± 9.9%, $p = 0.016$; 24 h: 21.9 ± 3.7% vs. 7.6 ± 1.3%, $p = 0.022$; 7 days: 18.3 ± 4.0% vs. 3.6 ± 1.9%, $p = 0.029$, $n = 3$ hearts/group).

LV remodeling and cardiac function after MSCs transplantation. At day 14 after cell transplantation, the thickness of infarct wall was significantly higher in the MSC_{HO-1} group compared with the other three groups (Figs. 3A and 3C). Furthermore, the percentage of fibrosis area in total LV area was significantly reduced in MSC_{HO-1}-engrafted mouse heart compared with the other groups (Figs. 3B and 3D). Left ventricular systolic performance and diastolic performance, as assessed by maximum dP/dt and minimum dP/dt, were significantly better in the MSC_{HO-1} group compared with MSC_{LacZ}, MSCs, and medium group, indicating that survival MSC_{HO-1} contribute to preserve systolic and diastolic functions of the infarct heart. The MSC_{LacZ}- and MSC-grafted hearts demonstrated less LV remodeling and better hemodynamic function in comparison with the medium group (Figs. 3E and 3F).

DISCUSSION

We have demonstrated that hypoxia-inducible HO-1 plasmid modification of MSCs enhances their survival in ischemic myocardium by real-time PCR assay. Moreover, we showed that the mechanism for enhancing grafted MSC survival is related to role of HO-1 in antiapoptosis and anti-inflammatory in ischemia myocardium. Mesenchymal stem cells treated with hypoxia-regulated HO-1 plasmid also attenuate LV remodeling and improve LV function, which is probably due to reduced apoptosis of grafted MSCs and reduced apoptosis in the host heart. These data suggest that hypoxia-regulated HO-1 plasmid gene modification of graft stem cells could be of significant value in improving the effectiveness of cell therapy in ischemic hearts.

The acute donor stem cell death that occurs immediately after engraftment is thought to have a major negative impact on the ensuing graft size and high level of cell death within four days after grafting into injured hearts (5). To reduce this attrition, the molecular mechanisms for the grafted cell death need to be identified and a graft cell death prevention strategy devised (19). Multiple mechanisms could contribute to death of graft cells. Recent reports suggest that graft cells placed in an ischemic environment were dependent on diffusion for the delivery of oxygen and substrates. In addition, host inflammatory response and loss of survival signal from cell-cell contact also may contribute to cell death (5). Other reports implicated the role of various proapoptotic or cytotoxic factors for molecular mechanisms of death of native or exogenous stem cells in the ischemic hearts (20). Implanted bone marrow mesenchymal stem cells seem to be highly sensitive to hypoxic and inflammatory environment in ischemic myocardium (6). Moreover, myocardial ischemia frequently is experienced in repeated bouts, which will

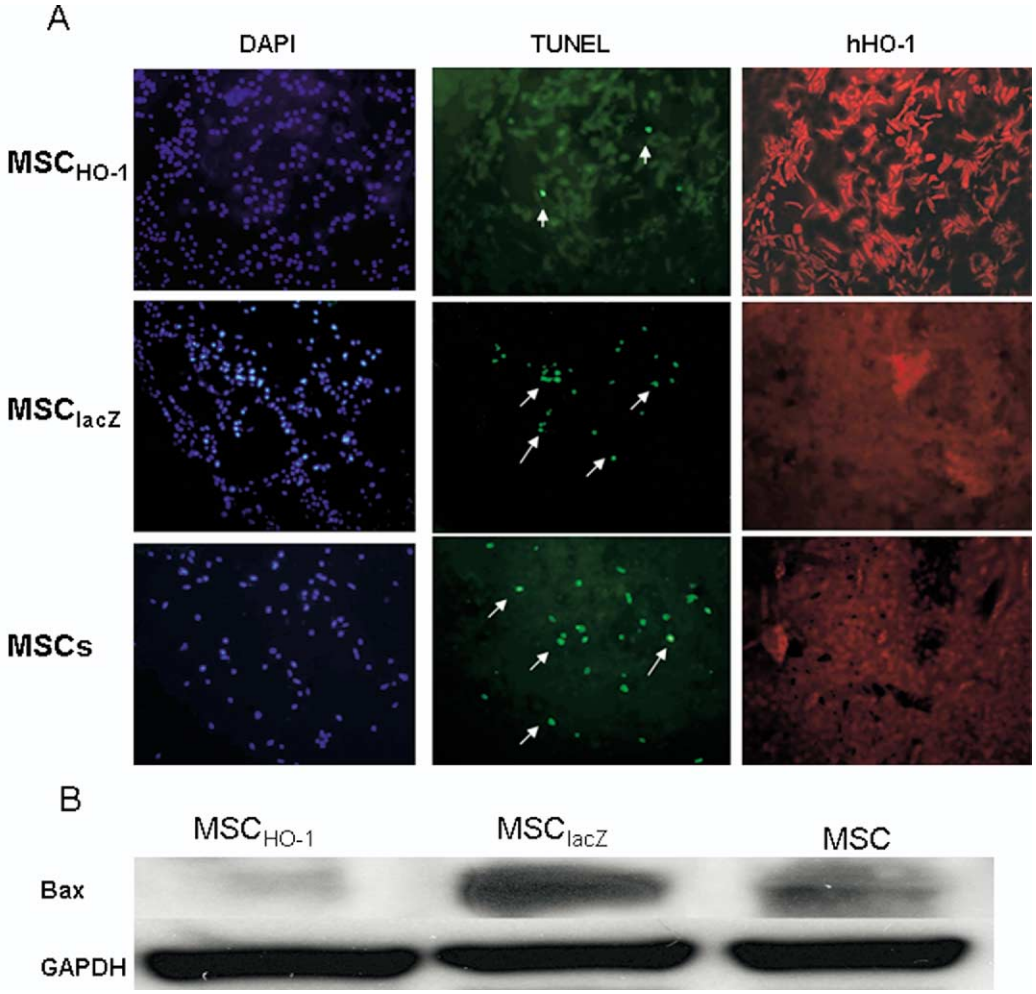

Figure 5. Effect of hypoxia-inducible hHO-1 treatment on protecting mesenchymal stem cell (MSC) against hypoxia/reoxygen damage in vitro. **(A)** Hypoxia-induced hHO-1 overexpression reduces MSC apoptosis in vitro by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate in situ nick end labeling assay. After hypoxia/reoxygen/hypoxia treatment, most of MSC_{HO-1} expressed human HO-1 in immunostaining whereas human HO-1 expression was low in MSC_{LacZ} or MSCs. The increase in human HO-1 expression in MSC_{HO-1} was accompanied by reduction in the cell apoptosis. **(B)** A down-regulation in a proapoptotic gene Bax level in the cell lysate of MSC_{HO-1} was confirmed by Western blot in comparison with MSC_{LacZ} and MSCs after hypoxia/reoxygen/hypoxia in vitro.

cause ischemic/reperfusion damage to implanted stem cells. There are currently debates on whether stem cells injected exogenously are engrafted to become new myocytes or if they act as a paracrine cells, secreting cardioprotective proteins. In either case, the protection of the transplanted MSCs with a gene modification, such as the hypoxic gene proposed here, will enhance survival, which will lead to improvement in cardiac function after transplantation of MSCs into infarct heart. Unknown numbers of newly regenerated cells may die by apoptosis during tissue remodeling and morphogenesis (20). Therapeutic application of gene-modified MSCs might require additional gene insertions with the ability to protect against ischemia in short-term and anti-ischemic/reperfusion damage in the long-term. Accordingly, these gene modifications would make the stem cells "vigilant." Vigilant stem cells would to be engineered with antiapoptosis, antisuperoxide, and anti-inflammatory genes for better survival in ischemic tissue and hence to enhance the efficiency of cardiac repair.

Several strategies aim at increasing cell survival after grafting. Suzuki et al. (21) reported that heat-shock treatment could improve cell tolerance to hypoxia-reoxygen insult in vitro and enhance survival when grafted into the heart. Exploiting cell growth and death regulatory factors to enhance the proliferation of viable stem cells or confer apoptosis resistance to donor cells also is used as a potential way to improve cell transplant efficiency. Mangi et al (22) demonstrated that a direct intramuscular injection of 5×10^6 Akt-engineered MSCs improved the function of infarct rat hearts. Akt is a powerful survival signal in many systems (23). However, the overall application of constitutively active Akt gene may increase the risk of tumorigenesis (24). Yang et al. (25) reported that HO-1 overexpression can prolong small-for-size allograft survival in liver by its anti-inflammatory action. Our study shows that overexpression of HO-1 in ischemic myocardium by direct plasmid injection decreased the number of infiltrating mononuclear cells, and down-regulated pro-inflammatory cytokines (IL-

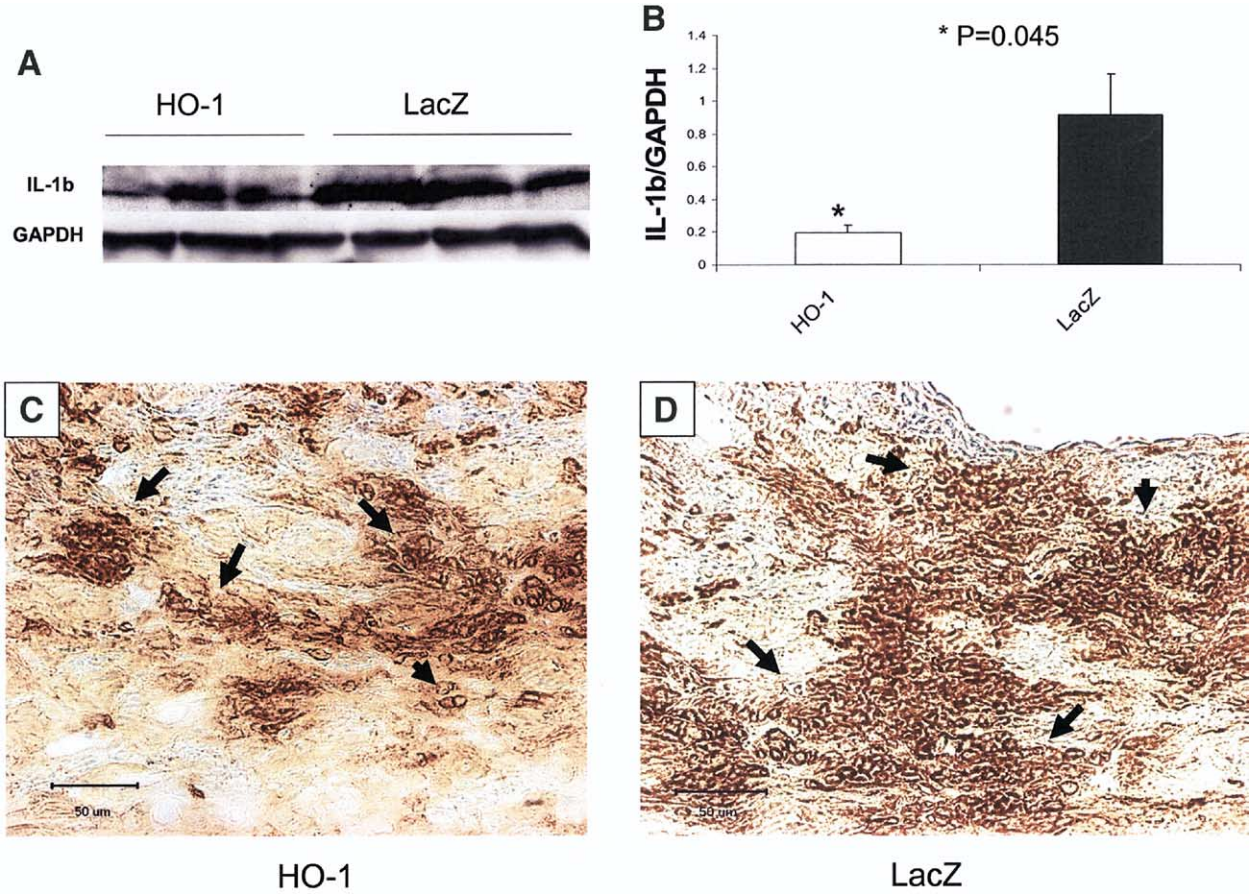

Figure 6. Detection of inflammatory cytokine by Western blot and inflammatory cell infiltration by CD45 staining in ischemic myocardium. **(A)** and **(B)** Down-regulation of interleukin-1β was detected in heme oxygenase-1 plasmid-treated ischemic myocardium one day after treatment, whereas an obvious elevation of interleukin-1b in LacZ plasmid-treated hearts was found. Data were shown as mean ± SE after normalization with glyseraldehyde-3-phosphate dehydrogenase and compared by Student *t* test, *p = 0.045 compared with LacZ. **(C)** and **(D)** Immunohistochemical staining to detect the CD45 cells in ischemic myocardium. The number of CD45-positive cells was decreased in HO-1 plasmid-treated myocardium four days after treatment. **Arrows** indicate the groups of CD45-positive cells.

1β). Similar results were also observed in the study of Melo et al. (12) on HO-1.

The possible mechanism of HO-1 action is related to phosphorylation of p38 mediated by carbon monoxide pathway (26). With our increased understanding of the key role of HO-1 in the adaptation and defense against cellular stress, HO-1 is a good candidate for providing a useful approach for cellular protection in ischemic heart through anti-inflammation and antiapoptosis mechanism (27). We have developed a hypoxia regulated vector system (8–10,15) for modulating tissue, which is based on an oxygen-dependent degradation domain from the hypoxia inducible factor-1-alpha. The OST can sense ischemia and switch on a cardioprotective gene (8–10,28). The present study demonstrates that inserting hypoxia-regulated HO-1 vector system into stem cells enhance their survival in ischemic hearts. The decrease in oxygen activates the hypoxia-inducible vector system expressing hHO-1 in these vigilant MSCs and improves their survival in cell culture and in vivo after engraftment to the ischemic myocardium. Mesenchymal stem cells modified in this manner could provide both positive paracrine activity and increased likelihood of survival to become graft cells for tissue regeneration. The vectors allow transgene expression in the regenerated tissue from graft cells to be regulated in response to a physiological signal, such as oxygen and to be switched on or off. The amplification of gene expression provides high enough levels to be effective.

We quantified the time course of graft cell survival for one week as the process of cell death appeared to be largely completed by seven days after grafting (5). Real-time PCR assay in gender-mismatched transplant provides us with an accurate and sensitive way to measure MSC engraftment in vivo. It avoids many biases in counting visible donor cells in random field from representative tissue sections (16). It is worthy to notice that there is a great discrepancy in reporting of graft cell survival. Some studies reported more than 90% of donor myoblasts died within first hour after transplantation in dystrophic mdx female mice (29) using a Y-chromosome–specific probe for blotting, whereas Muller-Ehmsen et al. (17) using real-time PCR and Zhang et al. (5) using TUNEL apoptosis assay both showed most cell death occurring one to two days after graft. Real-time PCR assay of Sry gene suggested that many cells were lost

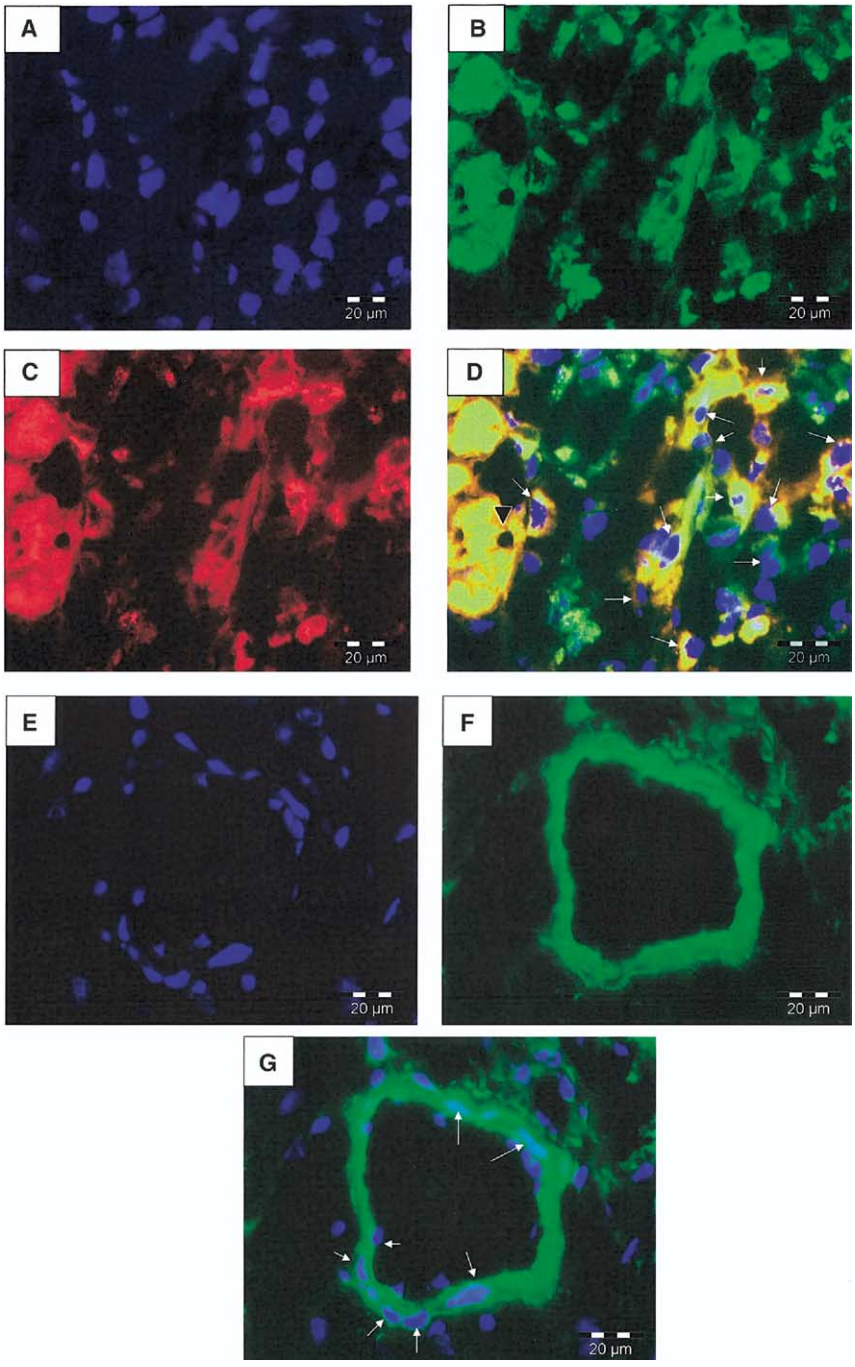

Figure 7. Differentiation of graft mesenchymal stem cell $(MSC)_{HO-1}$ in ischemic myocardium at 14 days after transplantation. 1) Cardiac muscle differentiation. **(A)** DAPI-labeled nuclei is shown in blue; **(B)** muscle marker α-actin in green; **(C)** cardiac marker cardiac troponin T in red; **(D)** Merged image of **(A)**, **(B)**, and **(C)** indicating that some graft cells adopt cardiac phenotype. **Arrows** point to the graft cell express cardiac mark and **arrowhead** indicates to host ischemic myocardium. 2: Endothelial cell differentiation. **(E)** DAPI-labeled nuclear; **(F)** endothelial marker CD31 in green; **(G)** Merged image of **(E)** and **(F)** indicating some graft cells participate in new microvessel formation.

within 24 h of transplantation, but 15% survived for 12 weeks (17). The quick loss during the first hour after implantation mainly is caused by cell retention in the syringe dead-space, leakage out of the myocardium, or wash-out through the vascular system, which can be controlled by improving transplantation method. This discrepancy may contribute to differences reported in donor cell type, animal model, and detection method. In our study, we combined real-time PCR with TUNEL apoptosis assay to provide reliable analysis. The hypoxia regulated HO-1 vector can express exogenous HO-1 earlier than endogenous HO-1 expression, as early as a half hour after ischemia (15), which has been confirmed by another report using a similar system (15,30). Overexpression of HO-1 in early

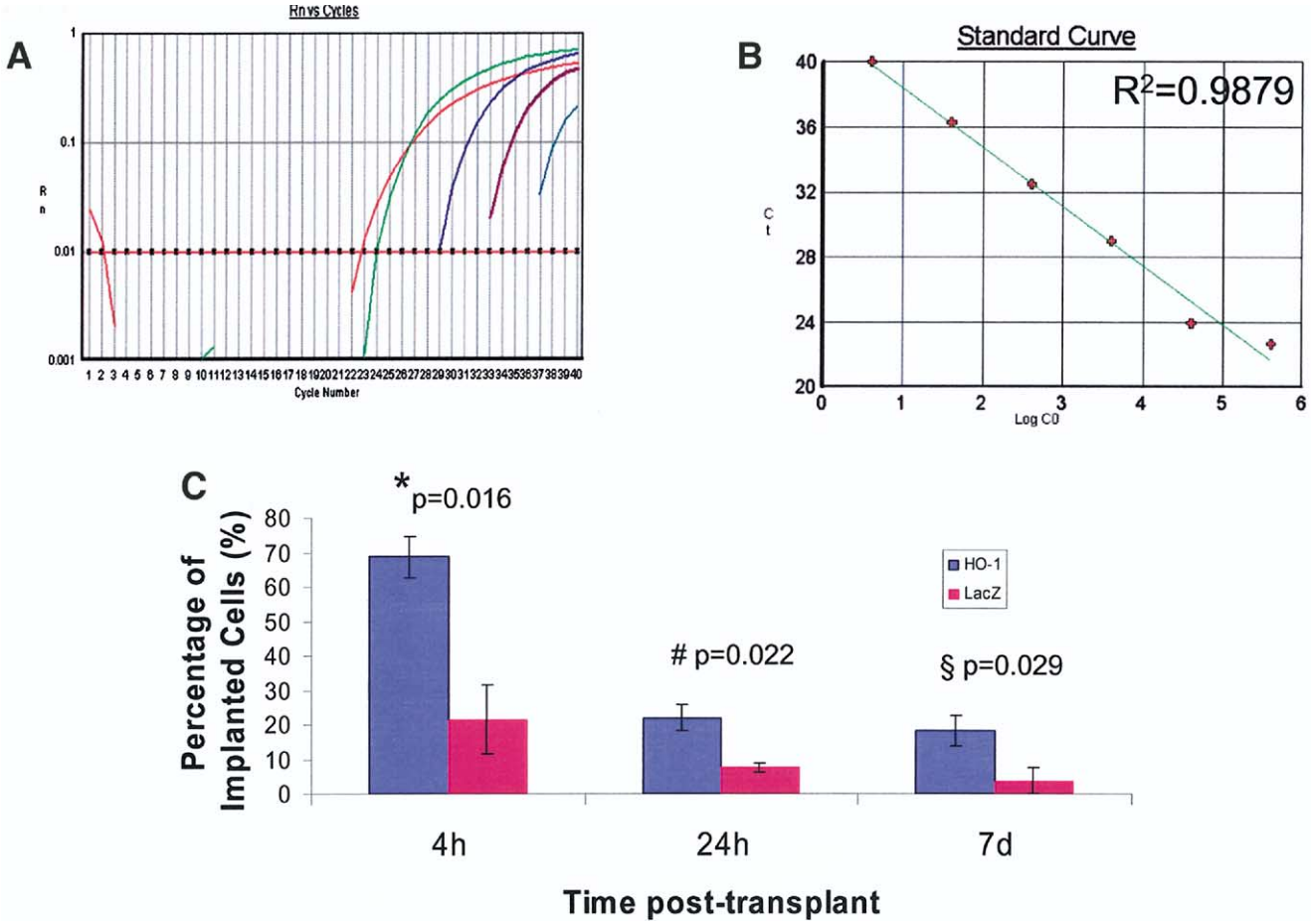

Figure 8. Quantifying graft male mesenchymal stem cells (MSCs) in the female ischemic myocardium by real-time polymerase chain reaction. **(A)** Real-time amplification plot showing change in normalized reporter dye fluorescence (Rn) versus number of amplification cycles in sample containing serially diluted male MSC genomic DNA. **(B)** Standard curve generated from data in **(a)** showing relationship between threshold cycle (Ct) and number of male MSCs. **(C)** Time course of graft MSC survival (%). The survival for MSC_{HO-1} was approximately 68% at 4 h and further decreased to 18% at seven days; however, the survival for MSC_{LacZ} decreased from 21% to 3.6% for this time period.

stages could better exert its beneficial effects on prolongation of graft cell survival.

Graft MSCs require vasculature to provide blood supply for their survival. It has been demonstrated that implanted MSCs can differentiate into endothelial phenotype and enhance vascular density as early as day 5 after implantation in rat myocardial infarction model (31,32). This short time window enables the use of plasmid vectors, which last only 10 to 14 days. Nonviral vectors are safe and efficient in protecting graft MSCs from short-term ischemic damage, which plays a critical role in graft stem cell death. In additional, hypoxia regulated HO-1 modification did not suppress the differentiation potential of engrafted MSCs. Our study shows cardiac muscle differentiation and endothelial cell differentiation of MSC_{HO-1} in ischemic myocardium. However, the mechanism of generating new myocytes and endothelial cells by MSCs is not clear, which may either from fusion or transdifferentiation (6,33), even at very lower frequency. More recently, we (34) and other groups (33) reported that graft stem cells can secrete several important survival factors, such as vascular endothelial growth factor, stromal cell-derived factor, basic fibroblast growth factor, hepatocyte growth factor, and insulin-like growth factor, which could protect or salvage endangered ischemic myocardium (35). This paracrine effect may be as important as the differentiation potential of stem cells for functional improvement.

Acute MI will cause severe ischemia, followed by an inflammation response, which significantly reduces the grafted cell survival rate. In this study, the rationale for choosing the acute phase of myocardial ischemia as the time for stem cell transplantation is based on following facts: 1) The paracrine action from graft stem cells can salvage ischemic myocardium at early phase, and 2) clinical application of stem-cell therapy currently exists in most large heart hospitals as a supplemental treatment to angioplasty patients with acute MI (36,37). Therefore, protecting graft cells from acute death in ischemic myocardium is important for clinical applications. However, the hypoxia-sensitive vector system only can be activated in an ischemic environment. Thus, a later treatment would not activate HO-1 and exert cardiac protection because as collateral circulation is rebuilt, the myocardial ischemia can be partial released. Moreover, a later application of the vector would produce a

less effective paracrine action of graft stem cells to salvage ischemic myocardium.

Conclusions. We have demonstrated that a hypoxia-inducible HO-1 plasmid modification of MSCs protects engrafted MSCs from apoptosis and inflammatory injury by the overexpression of HO-1 in ischemic myocardium. As a treatment, the transplantation of gene modified MSCs is associated with the improvement of LV function and ameliorate LV remodeling. Hypoxia-inducible HO-1 vector modification could be an effective graft cell death-prevention strategy. This evidence is the first using physiologically inducible expression of therapeutic genes for improving survival of graft stem cells in ischemic myocardium.

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