Hypoxia-inducible Vascular Endothelial Growth Factor-engineered Mesenchymal Stem Cells Prevent Myocardial Ischemic Injury

Sun Hwa Kim[1], Hyung-Ho Moon[1], Hyun Ah Kim[2], Ki-Chul Hwang[1], Minhyung Lee[2] and Donghoon Choi[1]

[1]Severance Integrative Research Institute for Cerebral & Cardiovascular Disease, Yonsei University Health System, Seoul, South Korea; [2]Department of Bioengineering, College of Engineering, Hanyang University, Seoul, South Korea

In the absence of repair mechanisms involving angiogenesis and cardiomyogenesis, loss of cardiomyocytes after myocardial injury is a primary causative factor in the progression toward heart failure. In an effort to reduce ischemic myocardial damage, we investigated the effects on infarcted myocardium of transplantation of genetically modified mesenchymal stem cells (MSCs) that specifically expressed vascular endothelial growth factor (VEGF) under hypoxic conditions. A hypoxia-inducible VEGF expression vector was introduced into MSCs (HI-VEGF-MSCs) using a nonviral delivery method, which were then used for the treatment of ischemic myocardial injury in rats. In HI-VEGF-MSCs, VEGF expression was significantly increased by hypoxia *in vitro* as compared to normoxia. Likewise, *in vivo* administration of HI-VEGF-MSCs induced ischemia-responsive VEGF production, leading to a significant increase in myocardial neovascularization after myocardial infarction. When compared with unmodified-MSCs, HI-VEGF-MSCs were retained in infarcted myocardium in greater numbers and remarkably reduced the number of apoptotic cells the infarcted area. Transplantation of HI-VEGF-MSCs resulted in a substantial attenuation of left ventricular remodeling in rat myocardial infarction. This study demonstrates that cell-based gene therapy using genetically engineered MSCs to express VEGF in response to hypoxic stress can be a promising therapeutic strategy for the treatment of ischemic heart disease.

Received 30 June 2010; accepted 12 December 2010; published online 18 January 2011. doi:10.1038/mt.2010.301

INTRODUCTION

Despite rapid advances in the medical treatment of cardiometabolic risk factors, cardiovascular disease remains the leading cause of morbidity and mortality in the world.[1] Since adult hearts have a limited capacity for regeneration, stem cell-based approaches for cardiac repair following myocardial infarction have been investigated and show considerable promise in reducing myocardial infarct size and improving cardiac function.[2] Recently, bone marrow-derived mesenchymal stem cells (MSCs) have attracted attention as an excellent model for development of cell-based therapeutics due to their potential for repairing damaged tissue.[3] In particular, MSCs possess many advantageous cellular attributes, such as ease of isolation, high expansion potential, genetic stability, and multipotential differentiation capacity.[4] For these reasons, MSCs have emerged as the most suitable candidates for use in cellular therapy and tissue engineering. Several preclinical studies have reported that MSCs positively modulate deleterious ventricular remodeling and improve recovery of cardiac performance after myocardial infarction,[5] although the exact repair mechanisms behind these effects have not yet been elucidated. Restoration of cardiac function after myocardial infarction requires not only replacement of lost cardiomyocytes but also neovascularization of ischemic myocardium. Emerging evidence suggests that MSCs are capable of regenerating both blood vessels and myocardium damaged after myocardial infarction.[4,6] Further, experimental studies evaluating the histological localization of MSCs have reported that a majority of engrafted MSCs are positive for cardiomyocyte-specific marker proteins, and that a distinct sub-population of transplanted cells are incorporated into microvessels at sites of revascularization.[7]

In spite of widespread interest in MSCs, an optimal approach for MSC-based tissue repair and regeneration has not been established. Indeed, poor viability and limited incorporation into target tissues are major drawbacks to achieving long-term therapeutic success in MSC therapy. Based on the research used to quantify the degree of MSC engraftment in ischemic myocardium, the survival rate of transplanted mouse MSCs is thought to be <1% at day 4 postimplantation.[4] Although administration of a higher number of cells can provide a small augmentation with respect to long-term engraftment, intact MSCs transplantation itself is not sufficient to alleviate progressive deterioration in myocardial function. To overcome these issues, cell therapy using genetically engineered cells has been suggested as an effective strategy to improve the survival rate and therapeutic efficacy of MSCs.[8–10] In addition to therapeutic applications in tissue regeneration, MSCs may also be useful as vehicles for *in vivo* gene delivery owing to their good genetic stability. After transfection with exogenous genes using various gene carriers, MSCs can sustain stable and

Correspondence: *Donghoon Choi, Severance Integrative Research Institute for Cerebral & Cardiovascular Disease, Yonsei University Health System, 250 Seongsanno, Seodaemun-gu, Seoul 120-752, South Korea. E-mail: cdhlyj@yuhs.ac*

long-term transgene expression without genetic modification even after *in vivo* differentiation.[11] Thus, MSC-mediated gene therapy has been investigated as an attractive option for the treatment of numerous diseases including myocardial infarction.

Gene therapy with angiogenic factors holds great promise in the treatment of ischemic diseases due to its ability to promote neovascularization.[12] In particular, vascular endothelial growth factor (VEGF), one of the most potent angiogenic factors, is capable of actively affecting both angiogenesis and myogenesis in cardiac injury repair.[13] More recent works have shown that VEGF treatment leads to enhancement of MSC viability in infarcted hearts by reducing cellular stress and increasing cell survival factors.[14,15] Thus, it is anticipated that cell therapy using genetically modified MSCs expressing VEGF may offer a potentially valuable approach for the treatment of myocardial infarction due to enhanced survival and angiogenic capacity. In our recent studies, we have constructed different types of hypoxia-specific VEGF expression systems and have used them as effective gene therapy strategies for salvaging myocardial ischemia and infarction due to their abilities to precisely controlled VEGF expression in the regions of ischemia.[16–19] Considering these findings, we hypothesized that the combination of two promising strategies, MSC transplantation and ischemia-inducible VEGF gene transfer, could induce successful MSC engraftment in ischemic myocardium through localized enhancement of VEGF levels, thereby maximizing their respective therapeutic potentials. In this study, we genetically modified rat MSCs using a hypoxia-inducible VEGF plasmid (pEpo-SV-VEGF). We then used these cells, which had enhanced cell survival, to promote angiogenesis in order to more effectively repair infarcted rat myocardium. We expected the hypoxia-inducible VEGF-engineered MSCs (HI-VEGF-MSCs) to overexpress VEGF specifically under ischemic conditions in order to provide valuable therapeutic benefits *in vivo*. We examined *in vitro* gene transfection efficiencies in rat MSCs with various nonviral carriers and VEGF plasmids to optimize the preparation of HI-VEGF-MSCs and then evaluated the therapeutic potentials of HI-VEGF-MSCs *in vivo* using a rat myocardial infarction model.

RESULTS

Preparation and characterization of HI-VEGF-MSCs

In vitro *transfection and cell viability assay.* In primary cultured cells including bone-marrow-derived MSCs, most transfection techniques achieve very low efficiencies, generally no more than a few percent. Thus, preparing effective transfection methodologies is crucial to the success of MSC-based gene therapy.[20,21] In order to provide optimal transfection efficiency in MSCs, commonly used nonviral transfection methods were evaluated in rat MSCs using pCMV-Luc as a reporter gene (**Figure 1**). Although genetic modification has been performed mainly using viral techniques, herein we excluded this method due to the concerns of

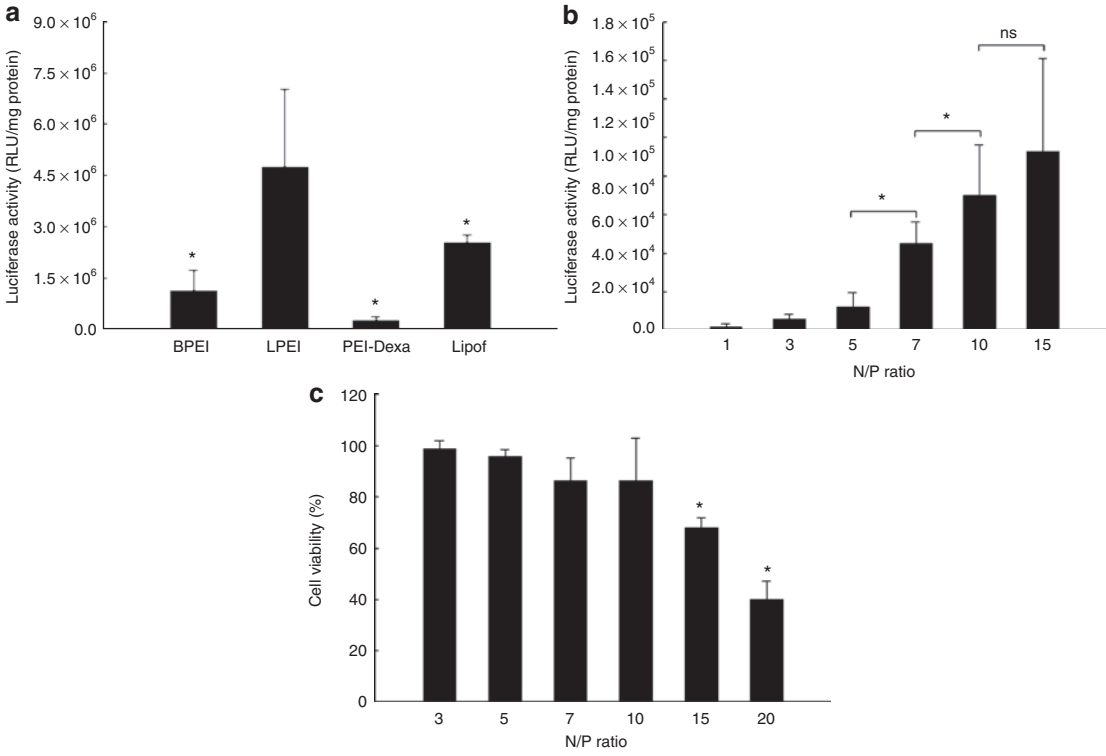

Figure 1 Plasmid transfection efficiency in mesenchymal stem cells (MSCs) using nonviral transfection reagents. (**a**) Selection of transfection reagent providing the best efficiency among conventional nonviral gene delivery vectors. pCMV-Luc plasmid (pDNA concentration = 0.2 μg/ml) was transfected into rat MSCs with branched poly(ethylenimine) (BPEI, 25 kDa), linear PEI (LPEI, 25 kDa), dexamethasone-conjugated BPEI (PEI-Dexa, 2 kDa), and Lipofectamine (Lipof) at transfection reagent-to-pDNA weight ratios of 1, 1, 8, and 12, respectively. *$P < 0.05$ versus LPEI. (**b**) Luciferase expressions in MSCs transfected by pCMV-Luc (pDNA concentration = 0.2 μg/ml) condensed with LPEI at various N/P (nitrogen of PEI/phosphate of DNA) ratios ranging from 1 to 15 (weight ratio from 0.1 to 2.0). *$P < 0.05$. (**c**) Cellular toxicities of LPEI/pDNA complexes prepared at various N/P ratios ranging from 3 to 20 in MSCs. *$P < 0.05$ versus N/P ratio of 3. RLU, relative light unit; ns, not significant.

insertional mutagenesis and immune response caused by viral vectors.[22] Four nonviral gene transfection reagents, branched poly(ethylenimine) (BPEI), linear PEI (LPEI), dexamethasone-conjugated PEI, and Lipofectamine, were examined in order to identify the best reagent for MSCs in terms of eliciting the highest transfection efficiency possible (**Figure 1a**). Branched PEI is the most widely studied nonviral gene transfer method despite its relatively high cytotoxicity.[23] Although LPEI shows slightly low transfection efficiency in some commonly used cell lines, it can be used as an alternative transfection method owing to the extremely low cytotoxicity.[23] Dexamethasone-conjugated PEI has been used previously to enhance the transfection efficiency of normal and cancer cell lines due to its high potency for translocation of polymer/pDNA complexes into the nucleus.[24]

Among the four nonviral vectors described above, LPEI exhibited the highest luciferase activity in rat MSCs, nearly twofold higher than that of the commercial transfection agent lipofectamine, which produced the second highest transfection efficiency. Based on this result, LPEI was used as the transfection reagent for MSCs in further experiments. To optimize the high-efficiency transfection methodology for low passage MSCs derived from rat bone marrow, the transfection efficiencies and cytotoxicities of polymer/pDNA complexes were estimated at varying mixing ratios of LPEI to pDNA (**Figure 1b,c**). Transfection efficiencies gradually increased with an increasing N/P ratio (nitrogen of PEI/phosphate of DNA), which plateaued at a N/P ratio of 10. Enhancement in gene expression was accompanied by a corresponding decrease in cellular viability; however, cytotoxicity was not observed up to a N/P ratio of 10. Thus, on the basis of these *in vitro* transfection results, the optimized transfection condition consisting of LPEI at a N/P ratio of 10 was used in the following experiments to evaluate the therapeutic potentials of HI-VEGF-MSCs both *in vitro* and *in vivo*.

In vitro *VEGF expression under hypoxia.* Levels of VEGF expression in genetically engineered MSCs under hypoxic conditions were examined using three different candidates for hypoxia-inducible VEGF plasmids (**Figure 2a**). Specifically, pSG5-VEGF, pRTP801-VEGF, and pEpo-SV-VEGF were transfected into MSCs, which were then cultured in hypoxic conditions (**Figure 2b**). The

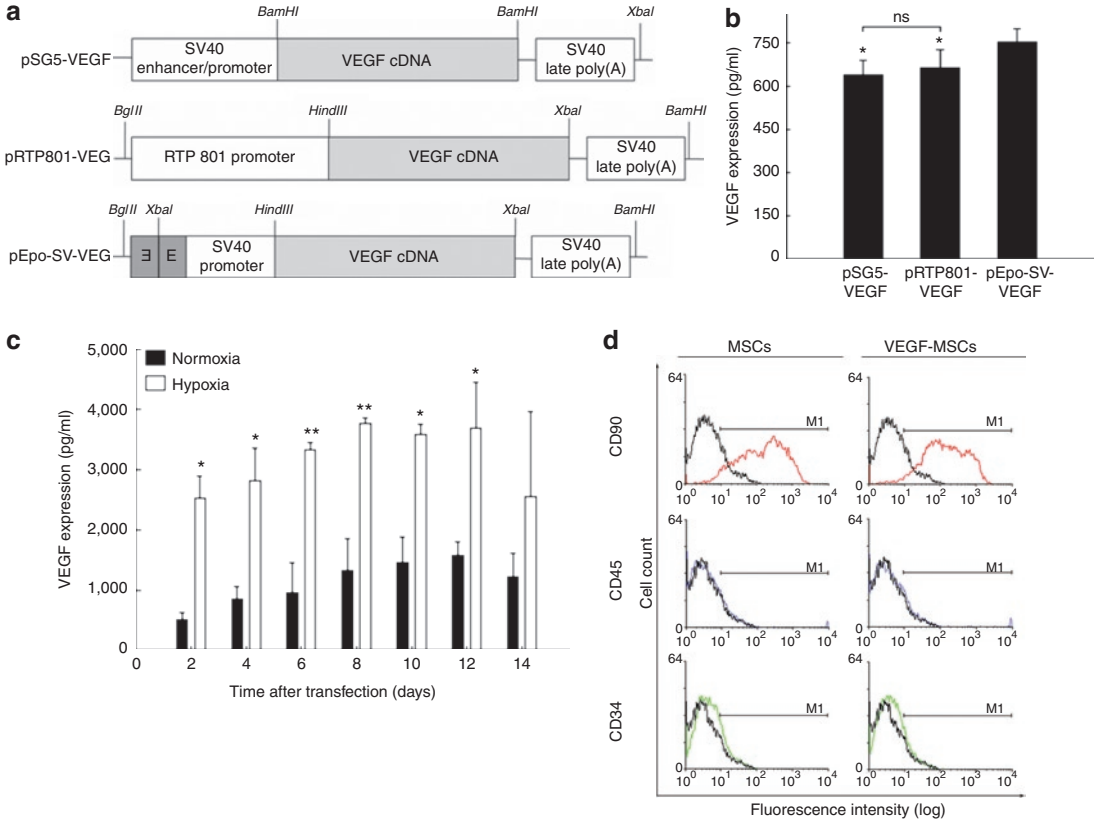

Figure 2 Preparation of hypoxia-inducible vascular endothelial growth factor-engineered mesenchymal stem cells (HI-VEGF-MSCs). (**a**) Schematic representation of the different types of hypoxia-inducible VEGF expression vector constructs. (**b**) Selection of hypoxia-inducible VEGF plasmid having the highest gene expression efficiency in MSCs. Each VEGF expression vector was transfected into MSCs with linear poly(ethylenimine) (N/P (nitrogen of PEI/phosphate of DNA) ratio = 10). After transfection, cells were incubated in hypoxic conditions for 1 day, and the amount of secreted VEGF in the culture supernatant was determined with enzyme-linked immunosorbent assay (ELISA). *$P < 0.05$ versus pEpo-SV-VEGF. (**c**) Level of VEGF expression in HI-VEGF-MSCs under normoxia and hypoxia. pEpo-SV-VEGF was transfected into MSCs with LPEI (N/P ratio = 10). After transfection, cells were further incubated in normoxic or hypoxic conditions for the desired time period. The VEGF concentration of each conditioned medium was measured using ELISA. *$P < 0.05$ versus normoxia; **$P < 0.01$ versus normoxia. (**d**) Phenotypic analysis of MSCs surface markers. MSCs and HI-VEGF-MSCs were stained for different selectable markers (positive: CD90 (red), negative: CD45 (blue) and CD34 (green)). An unstained MSCs sample was used as a negative control (black). M1 refers to the gated area (fluorescence intensity (arbitrary unit): 10^1–10^4). ns, not significant.

simian virus 40 (SV40) enhancer-promoter, RTP801 promoter, and erythropoietin enhancer-SV40 promoter were previously developed to induce target gene expression at the transcriptional level in response to hypoxic stresses *in vitro* and *in vivo*.[16,18] Of the three hypoxia-inducible VEGF expression vectors, pEpo-SV-VEGF showed the highest level of VEGF expression in MSCs under both hypoxia and normoxia (data not shown). Although intact MSCs are known to produce VEGF under normoxic and hypoxic conditions,[25] the VEGF levels in plain MSCs fall below the range of detection by an enzyme-linked immunosorbent assay (ELISA) kit in both normoxia and hypoxia (data not shown). Based on the potency in activating hypoxic gene expression, the pEpo-SV-VEGF vector was used to generate HI-VEGF-MSCs for further *in vivo* applications. In order to assess the impact of persistent hypoxic exposure on VEGF gene expression levels in MSCs, the pEpo-SV-VEGF-transfected MSCs were incubated under hypoxia and normoxia for the desired period of time from 2 to 14 days (**Figure 2c**). When pEpo-SV-VEGF-transfected MSCs were exposed to hypoxic conditions, cells maintained a constant and high level of VEGF expression compared to those of cells incubated in normoxia. The elevated levels of VEGF expression in HI-VEGF-MSCs lasted at least 2 weeks. It is generally accepted that nonviral vector-mediated transient transgene expression can be sustained for up to 4 weeks in primary cells.[26] In addition, previous studies have reported that VEGF-engineered MSCs increased VEGF induction within 1 day after transplantation on ischemic myocardium and the enhancement was maintained until 1 to 2 weeks and then disappeared. These prompted us to estimate the *in vivo* therapeutic effectiveness of HI-VEGF-MSCs in rat ischemic myocardium 2 weeks postimplantation. Importantly, as shown in **Figure 2d**, the expression pattern of MSCs surface markers did not change after pEpo-SV-VEGF modification.

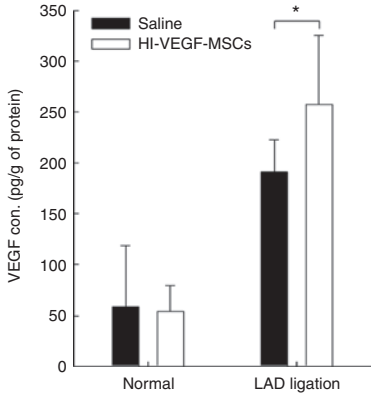

Figure 3 ***In vivo*** **vascular endothelial growth factor (VEGF) expression levels of hypoxia-inducible-VEGF-engineered mesenchymal stem cells (HI-VEGF-MSCs) in normal and ischemic myocardium.** HI-VEGF-MSCs were transplanted into myocardium with/without left anterior descending coronary artery-ligation (n = 10/group). Control groups received saline injections (n = 6/group). Tissue samples were collected 2 days after transplantation and were analyzed by enzyme-linked immunosorbent assay. Normal refers to sham-operated myocardium. *P < 0.05.

In vivo HI-VEGF-MSC transplantation in ischemic myocardium

In vivo *VEGF expression*. To estimate whether HI-VEGF-MSCs expressed VEGF in response to ischemia *in vivo*, myocardial VEGF levels after cell transplantation were analyzed in normal and left anterior descending coronary artery (LAD)-ligated animals (**Figure 3**). In normal heart samples, VEGF expression in HI-VEGF-MSC-transplanted groups (54.5 ± 25.4 pg/g of protein) was maintained at levels similar to that of the control group (58.5 ± 60.7 pg/g of protein). It is particularly interesting to note that the HI-VEGF-MSCs transplantation (257.7 ± 67.4 pg/g of protein) showed ~1.4-fold increase in VEGF production in LAD-ligated hearts compared with that of saline treatment (191.1 ± 31.2 pg/g of protein), in good agreement with our *in vitro* transfection experiments. Unfortunately, serum VEGF levels were below the range of detection using an ELISA kit in both the normal animals and the LAD-ligation model (data not shown), likely

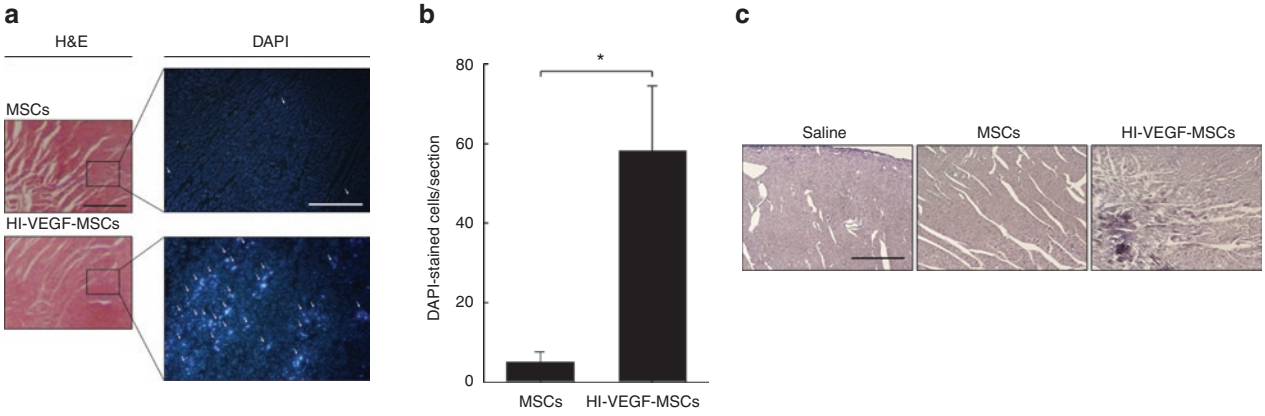

Figure 4 Engraftment efficiencies of hypoxia-inducible vascular endothelial growth factor-engineered mesenchymal stem cells (HI-VEGF-MSCs) within ischemic myocardium. (**a**) Representative hematoxylin and eosin (H&E, left panel, ×100) and 4′,6-diamidino-2-phenylindole (DAPI, right panel, ×400) stained myocardial sections. Tissue samples were obtained from left anterior descending coronary artery (LAD)-ligated rats 3 days after injection of MSCs and HI-VEGF-MSCs. The presence of transplanted MSCs was recognized by blue fluorescence signals generated by DAPI (white arrow). Bars: black = 500 µm, white = 100 µm. (**b**) Comparison of engraftment levels of MSCs and HI-VEGF-MSCs in ischemic myocardium (n = 6/group). *P < 0.01. (**c**) Immunostaining of human VEGF. Tissue samples were obtained from LAD-ligated rats at 2 days after transplantation and were stained with antihuman VEGF antibodies. ×100 magnification, Bar = 500 µm.

due to the rapid diffusion of secreted VEGF into the serum in high-dilution conditions.

MSC engraftment and human VEGF expression. For histological tracking of implanted MSCs, the presence of 4′,6-diamidino-2-phenylindole (DAPI)-labeled MSCs and HI-VEGF-MSCs were analyzed after experimentally induced myocardial infarction in rats (**Figure 4c,d**). Upon injection of unmodified MSCs into the infarcted heart, only a few DAPI-positive cells were detected in the infarcted area 3 days postimplantation; however, when HI-VEGF-MSCs were injected, there was a marked increase in the number of DAPI-positive cells. As shown in **Figure 4b**, the achieved engraftment efficiencies of MSCs and HI-VEGF-MSCs were 5.0 ± 2.6 and 58.3 ± 16.2 cells per section, respectively. In general, the number of engrafted MSCs is reported to be virtually diminished within 3–5 days after implantation.[27] In particular, HI-VEGF-MSC-transplanted rat myocardium exhibited a distinct immuno-reactivity against the antihuman VEGF antibody (**Figure 4c**), suggesting that the HI-VEGF-MSC engraftment can provide additional human VEGF as well as rat VEGF induced by myocardial ischemia in rats (**Figure 3**).

Neovascularization. To examine the density of capillaries in the infarcted heart after cell transplantation, immunostaining of micro-vessels using a CD31 antibody was performed for ischemic rat myocardium 2 weeks post-transplantation (**Figure 5a,b**). As compared to saline and intact MSC-injected groups, HI-VEGF-MSC-transplanted groups exhibited a significant increase in microvascularization of the infarcted myocardial tissues. The mean number of CD31-positive cells per square millimeter of infarct was also quantified as shown in **Figure 5b**. Specifically, the micro-vessel densities in the infarcted myocardia treated with saline, MSCs, and HI-VEGF-MSCs were 75.0 ± 33.7, 157.9 ± 59.4, and 631.1 ± 171.3 $CD31^+$ cells/mm^2, respectively. The increase in vessel density appeared to be the result of VEGF induction in hypoxic MSCs, which is consistent with the expectation that high VEGF production may promote neoangiogenesis.[28]

Antiapoptotic effects in ischemic myocardium. To investigate the effect of cell transplantation on apoptotic cell death in the areas adjacent to the infarction, sections from saline-, MSC-, and HI-VEGF-MSC-treated hearts were processed for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay 2 weeks following LAD-ligation (**Figure 5c,d**). The degree of apoptosis was quantified by counting the number of TUNEL-positive cells in the sections (**Figure 5d**). The extent of TUNEL-positive apoptotic cell death was greatly increased after LAD-ligation (57.3 ± 10.3 $TUNEL^+$ cells/high power fields (HPF)). Both MSC-based treatments resulted in a trend toward reduction in apoptosis in the infarcted myocardium. Specifically, compared with the LAD-ligation group, intact MSC transplantation showed only 2.7-fold reduced induction of apoptosis (21.5 ± 5.4 $TUNEL^+$ cells/HPF), while ischemia-induced apoptotic cell death was attenuated up to 13.9-fold through HI-VEGF-MSC transplantation into infarcted myocardium (4.1 ± 0.5 $TUNEL^+$ cells/HPF). These results indicate that the improvement in VEGF expression

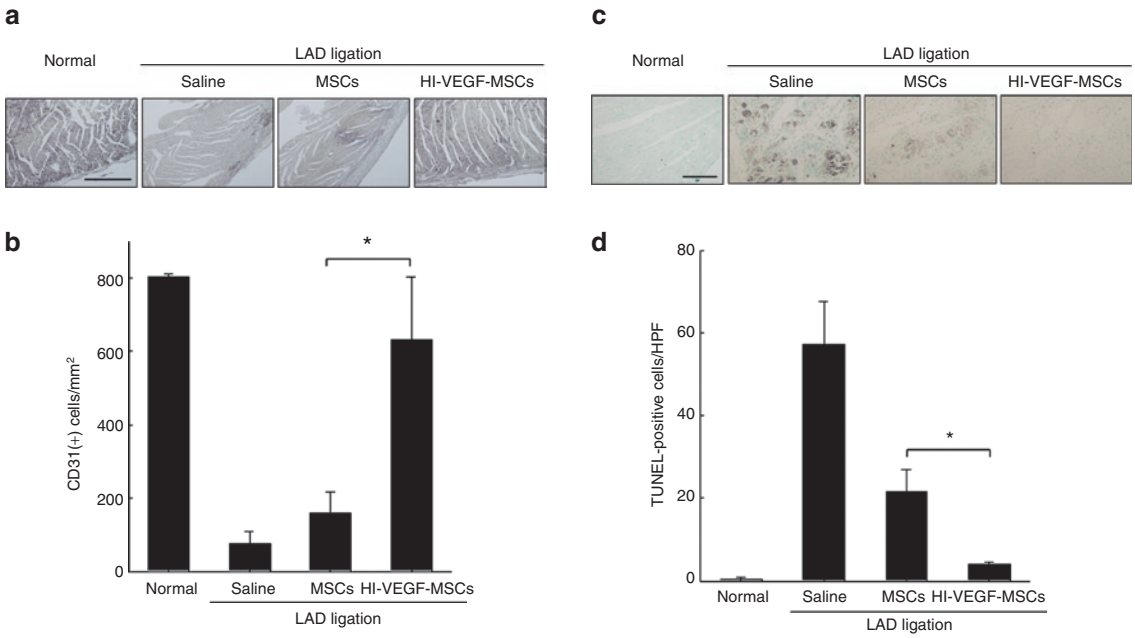

Figure 5 Effects of hypoxia-inducible vascular endothelial growth factor-engineered mesenchymal stem cell (HI-VEGF-MSC) transplantation on neovascular formation and apoptosis in ischemic myocardium. (**a**) Representative images of myocardial sections stained for CD31 (×100). Rat ischemic myocardia were injected with saline, MSCs, or HI-VEGF-MSCs. Normal refers to sham-operated myocardium. Tissue samples were collected 2 weeks after transplantation and were immunostained with anti-CD31. Bar = 500 µm. (**b**) Extent of capillary tube formation in ischemic myocardia treated with saline, MSCs, or HI-VEGF-MSCs (n = 6/group). *P < 0.01. (**c**) Representative micrographs of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-stained ischemic myocardium (×400). Rat hearts treated with saline, MSCs, and HI-VEGF-MSCs were stained with TUNEL reagents 2 weeks after transplantation. Bar = 100 µm. (**d**) Levels of apoptosis in ischemic myocardium treated with saline, MSCs, or HI-VEGF-MSCs (n = 6/group). *P < 0.01; HPF, high-power field.

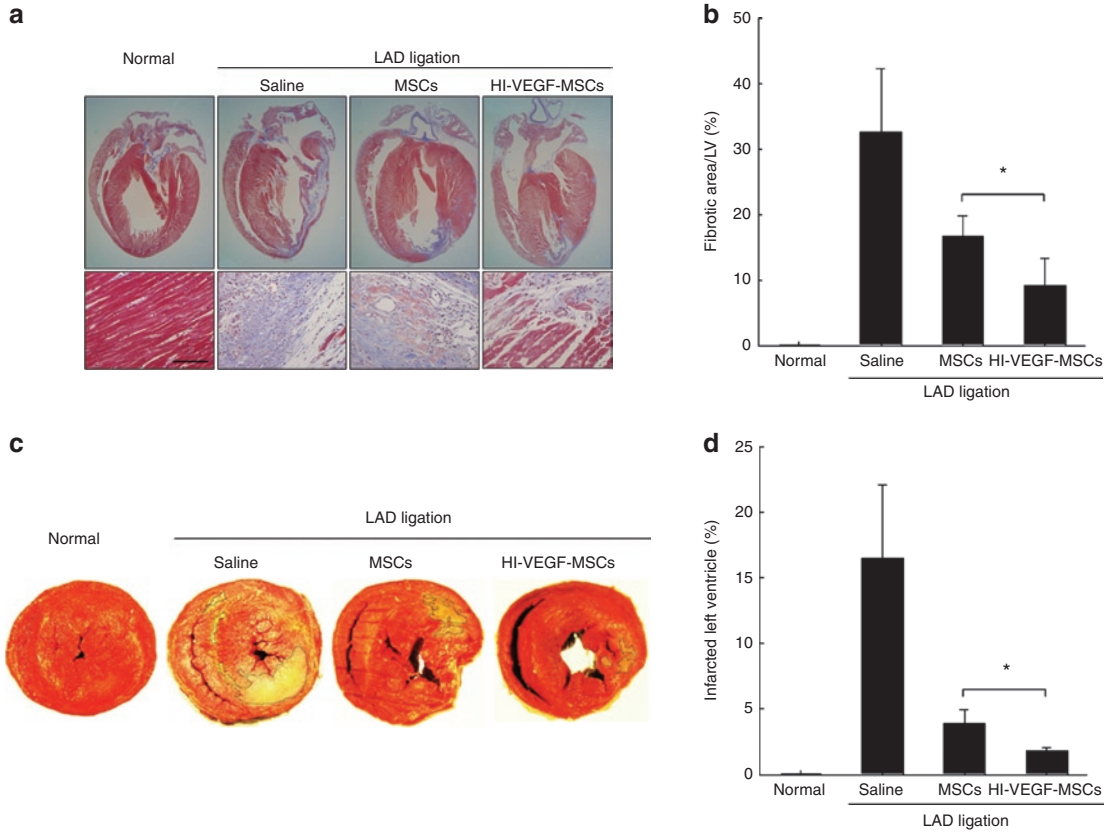

Figure 6 Effects of hypoxia-inducible vascular endothelial growth factor-engineered mesenchymal stem cell (HI-VEGF-MSC) transplantation on left ventricular fibrosis and infarct size after myocardial ischemia. (**a**) Representative histological sections of rat myocardium stained with Masson's trichrome (lower panel: ×200). Rat ischemic myocardia were injected by saline, MSCs, or HI-VEGF-MSCs. Normal refers to sham-operated myocardium. Tissue samples were collected 2 weeks after transplantation and were stained with hematoxylin and eosin and Masson's trichrome. Bar = 200 μm. (**b**) Percent fibrosis (myocardial collagen) expressed as the ratio of fibrotic area to left ventricle area in left anterior descending coronary artery (LAD)-ligated rats treated with saline, MSCs, or HI-VEGF-MSCs injections (n = 6/group). *P < 0.05. (**c**) Representative 2, 3, 5-triphenyltetrazolium chloride-stained sections of rat hearts (with/without LAD-ligation) with saline-, MSC-, or HI-VEGF-MSC-treatments. (**d**) Percentage of the ratio of infarcted to noninfarcted left ventricular myocardium from LAD-ligated rats treated with saline, MSCs, or HI-VEGF-MSCs (n = 6/group). *P < 0.01. LV, left ventricle.

by HI-VEGF-MSCs may have enhanced the ability to inhibit ischemia-induced apoptosis in myocardium after infarction.

Fibrosis and infarct size. The extent of cardiac fibrosis was assessed by staining collagen-containing fibers in rat myocardial tissue using Masson's trichrome. **Figure 6a** shows representative photographs of the longitudinal section of the heart 2 weeks following sham operation or LAD-ligation with/without cell transplantation. As shown in **Figure 6b**, the improvement in cardiac fibrosis was quite remarkable in HI-VEGF-MSC-transplanted groups (9.2 ± 4.1%) compared to those of the saline- and MSC-injected groups (32.5 ± 9.8 and 16.7 ± 3.2%, respectively). As shown by the representative photographs in **Figure 6c**, the infarcted fibrotic tissue appeared as a pale yellow color. Both MSC and HI-VEGF-MSC transplantations led to decrease in infarct size compared with that of the saline-treated control group. Infarct size was quantitatively assessed by measuring the ratio of infarcted myocardium in the left ventricle (**Figure 6d**). The degree of infarct expansion was approximately twofold lower in the HI-VEGF-MSC-transplanted animals (1.8 ± 0.3%) than it was in the unmodified MSC-treated animals (3.9 ± 1.1%), suggesting that the improved therapeutic effects of HI-VEGF-MSCs could be caused by their augmented neovascularization and cell survival, as observed above.

Cardiac function. To investigate the cardiac functional improvement of ischemic myocardium with HI-VEGF-MSC treatment, transthoracic echocardiography was performed at baseline and for saline-, MSC-, and HI-VEGF-MSC-transplanted rat ischemic myocardium (**Figure 7**). Two weeks after LAD-ligation, both MSC and HI-VEGF-MSC treatments improved cardiac function compared with the saline-injected groups (27.6 ± 6.5 and 32.2 ± 10.0% fractional shortening, and 50.7 ± 8.7 and 64.4 ± 16.9% ejection fraction, respectively). Although HI-VEGF-MSCs exhibited a higher level of myocardial functional recovery than MSCs, their enhancement of systolic performance was insufficient to show a statistically significant benefit in left ventricular functional performance at 2 weeks after LAD ligation.

DISCUSSION

Myocardial infarction frequently leads to deleterious remodeling of myocardium resulting in severe ventricular dysfunction.[4] Due to the limited regenerative capacity of the heart, several

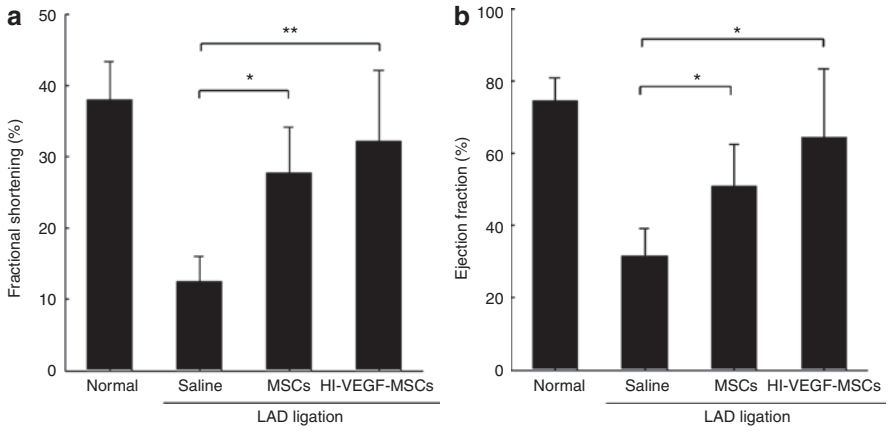

Figure 7 Effects of hypoxia-inducible vascular endothelial growth factor-engineered mesenchymal stem cell (HI-VEGF-MSC) transplantation on left ventricular function. (**a**) Left ventricular fractional shortening and (**b**) ejection fraction. Rat ischemic myocardia were injected by saline, MSCs, or HI-VEGF-MSCs (n = 6/group). Normal refers to sham-operated myocardium. Cardiac functions were assessed by transthoracic echocardiography 2 weeks after left anterior descending coronary artery (LAD) ligation. *P < 0.05; **P < 0.01.

approaches using autologous stem cells have been tested as a means to minimize myocardial ischemia.[2] Transplantation of bone marrow-derived MSCs has been shown to be effective in attenuating deleterious ventricular remodeling and improving cardiac performance after myocardial infarction.[29,30] However, MSC-based therapeutic strategies have achieved limited success in repairing damaged myocardium because of poor cell viability and low cell retention. Several recent reports have indicated that exogenous supplementation of angiogenic molecules such as VEGF and insulin-like growth factor 1 promotes MSC retention and growth in the myocardium after transplantation,[14,31] which are needed for successful long-term cardiac regeneration. In addition to their important role in enhancing the survival of MSCs, therapeutic angiogenesis induced by angiogenic factors has great potential in the prevention and treatment of ischemic heart disease. Accordingly, combining MSCs and angiogenic factors may provide a valuable therapeutic approach to improve the efficacy of cell-based treatments.

This study aimed to evaluate the effects of HI-VEGF-MSC transplantation on vascular regeneration and cardioprotection in a rat myocardial infarction model. In our recent studies, several hypoxia-specific VEGF expression systems were designed to be used as potential candidates for gene therapy in the treatment of ischemic limb and heart disease.[16–19] In order to facilitate VEGF production in MSCs especially under ischemic conditions, here in rat MSCs were genetically modified by transfection with a hypoxia-inducible VEGF plasmid (pEpo-SV-VEGF) using LPEI as a nonviral vector. There were no phenotypic changes in MSCs upon the hypoxia-inducible VEGF plasmid modification, which was determined by observing no marked differences in the expression patterns of positive (CD90) and negative (CD34 and CD45) selectable markers in MSCs after genetic modification (**Figure 2d**). Previous studies revealed that genetically engineered MSCs can maintain transgene expression without genetic modification *in vitro* and *in vivo*.[11] This genetic stability is one of the positive attributes of MSCs for development of cell-mediated gene therapy.

Both *in vitro* and *in vivo* exposures to hypoxic conditions displayed significantly higher levels of VEGF expression in HI-VEGF-MSCs compared to those experiencing normoxia (**Figures 2** and **3**). It should be noted that the transplantation of HI-VEGF-MSCs into normal heart sustained a low level of VEGF within the normal range. Thus, it was expected that the therapeutic approaches using HI-VEGF-MSCs would alleviate the safety concerns for VEGF-based therapeutics, such as risk of disease progression and mortality in cancer patients. Moreover, the control group with saline injection exhibited somewhat higher VEGF levels after LAD-ligation, indicating that hypoxia alone could induce VEGF production and angiogenesis in ischemic myocardia. It is well known that the local levels of VEGF temporarily increase in ischemic regions in order to support the body's defense mechanisms against further ischemia-reperfusion injury.[32] However, this slight increase in VEGF production in the ischemic heart is not sufficient to promote neovascularization or to attenuate myocardial ischemic injury through VEGF-induced angiogenic activities.[33]

Notably, the use of HI-VEGF-MSCs was able to promote transplanted cell retention and survival within the infarcted myocardium compared with that of unmodified MSCs (**Figure 4c,d**). In general, intact MSCs show no engraftment or only very low level engraftment. Several lines of experimental evidence have indicated that >97% of MSCs administered via direct intramuscular injection are typically eliminated from an infarct region after 2 weeks.[4] However, recent studies have revealed that engineered cells displaying increased VEGF levels exhibit enhanced cell survival and engraftment following injection into the target tissues compared to those of cells expressing low levels of VEGF. For instance, transplantation of VEGF-engineered myoblasts into mouse muscle led to increased VEGF levels and cell viability, resulting in improvements in skeletal muscle repair.[34,35] Therefore, it is conceivable that the increased cell survival rate of HI-VEGF-MSCs could be attributed to their remarkable induction of VEGF expression in response to ischemia. More recent studies have suggested that cardiac cell therapy provides therapeutic benefits mainly through the paracrine actions of factors released by transplanted cells, rather than by direct regeneration.[36,37]

It is interesting that transplantation of HI-VEGF-MSCs resulted in a significant reduction in ischemic apoptosis in the

region adjacent to the infarct myocardium (**Figure 5a,b**). This effect was most likely due to expression of high levels of VEGF mediated by ischemic stress, consistent with the findings of a previous studies demonstrating that VEGF prevents stress-induced apoptosis of muscle and endothelial cells.[38] Further, it has been observed that intramuscular administration of VEGF protein leads to reduced apoptosis in ischemic mouse skeletal muscle compared to that of control mice that did not receive VEGF.[39] In particular, engrafted HI-VEGF-MSCs dramatically promoted micro-vessel formation in rat myocardium after LAD-ligation (**Figure 5c,d**), mainly due to VEGF-induced capillary angiogenesis. In addition to their angiogenic effects, the use of HI-VEGF-MSCs was expected to provide myocardial protection against ischemic injury. It has been previously reported that the reduction in postmyocardial infarction remodeling is accompanied by an increased capillary density induced by VEGF.[40] Thus, VEGF induction by HI-VEGF-MSCs may contribute to increased capillary density in ischemic hearts, resulting in a remarkable reduction in infarct extension and fibrotic tissue formation (**Figure 6**). Since the extent of cardiomyocyte differentiation of transplanted stem cells was relatively meager, improvement in cardiac function from MSC therapy after myocardial infarction may not have been due to a significant replacement of lost cells. The augmentation of cardioprotective effects of HI-VEGF-MSC transplantation could be attributed to angiogenic and paracrine effects rather than direct replacement of lost cardiomyocytes from MSCs. Unfortunately, these robust enhancements of HI-VEGF-MSC treatment in various histologic findings were insufficient to show a statistically significant difference in cardiac functional performance, although rat ischemic myocardium injected with HI-VEGF-MSCs displayed a higher functional recovery compared to that injected by MSCs alone (**Figure 7**). It is probably due to the lack of an appropriate VEGF plasmid delivery system for primary cells such as MSCs. Since the therapeutic efficiency of genetically engineered cells mainly depends on the gene delivery vectors,[41] the development of technologies for efficient delivery of therapeutic genes into MSCs may provide more functional benefits. For potential clinical application of HI-VEGF-MSCs, use of enhancing gene delivery reagents need to be further considered.

In conclusion, we have demonstrated a new approach to cell-based gene therapy using MSCs generically engineered to overexpress VEGF under hypoxia for the treatment of myocardial infarction in rats. The transplantation of HI-VEGF-MSCs was able to enhance neovascularization, while simultaneously reducing apoptosis in infarcted myocardium through VEGF induction in response to ischemia. The enhanced and prolonged efficacy of MSC-based gene therapy using HI-VEGF-MSCs may provide beneficial effects in the attenuation of infarct expansion and the preservation of left ventricle area after myocardial infarction. Therefore, HI-VEGF-MSC transplantation may offer potential for a novel therapeutic strategy for the treatment of a number of clinical disorders related to ischemia-induced tissue injury, including stroke, coronary artery disease, and peripheral artery disease. Since the insufficient myocardial functional recovery of HI-VEGF-MSC treatment may restrict its clinical application, use of potential gene delivery systems should be further considered to provide optimal VEGF-engineered cells.

MATERIALS AND METHODS

Animals. Sprague–Dawley rats were obtained from Samtako Bio (Osan, Korea). The use of animals was in accordance with the International Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Animal Research Committee of Yonsei University College of Medicine.

MSC isolation and culture. Four-week-old male Sprague–Dawley rats (100 ± 5 g) were used for MSC isolation. MSCs were harvested, propagated, and characterized as described previously.[42,43] Briefly, rat bone marrow-derived cells were flushed out from femurs and tibias with culture medium. Mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). After 48 hours incubation, nonadherent cells were discarded, and adherent cells were further expanded until confluent. MSCs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin up to 80% confluency for three or four passages before their use *in vitro* and *in vivo*. In order to verify that the isolated cells were phenotypically MSCs, the cultured cells were labeled against various cell-surface markers characteristic for MSCs and analyzed by flow cytometry.

DNA construction. pCMV-Luc was constructed by inserting the firefly luciferase cDNA fragment from pGL3-control vector (Promega, Madison, WI) into the *HindIII*/*XbaI* sites of pcDNA3 (Invitrogen, Carlsbad, CA).[44] Since human VEGF165 is also responsible for the therapeutic and physiological effects in rats,[45] human VEGF165 cDNA was used for the construction of various hypoxia-inducible VEGF plasmids including pSG5-VEGF, pRTP801-VEGF, and pEpo-SV-VEGF. pSG5-VEGF was kindly provided by Jozef Dulak (Department of Cardiology, University of Innsbrook, Innsbrook, Austria). pRTP801-VEGF was generated by cloning the RTP801 promoter into pSV-VEGF as previously described.[16] Briefly, the human VEGF165 cDNA was amplified by PCR using pSG5-VEGF as a template. The *HindIII*/*XbaI* sites were attached at each end of the cDNA. The PCR product (VEGF cDNA) was inserted into the pSV-Luc vector (Promega) at the *HindIII*/*XbaI* sites after the deletion of the luciferase gene in order to construct pSV-VEGF. The RTP801 promoter was cloned using genomic DNA extracted from liver hepatocellular carcinoma cells with DNeasy Tissue kit (Qiagen, Valencia, CA). The *BglII*/*HindIII* sites were introduced into the RTP801 promoter. The SV40 promoter was then removed from pSV-VEGF and the PCR-amplified RTP801 fragment was inserted into the *BglII*/*HindIII* sites, to produce the pRTP801-VEGF vector. The pEpo-SV-VEGF vector was constructed as previously reported.[18] To produce the pEpo-SV-VEGF vector, the erythropoietin enhancer containing the *BamHI*/*XbaI* sites was synthesized chemically and was inserted at a *BglII* site upstream of the SV40 minimal promoter. Construction of plasmids was confirmed using restriction enzyme digestion and DNA sequence analysis.

In vitro *transfection.* For *in vitro* transfection study, MSCs were plated at a density of 1.0×10^5 cells/well in a six-well plate. After 24 hours of incubation, the culture medium was exchanged with fresh serum-free medium. Cells were transfected with the plasmids prepared with LPEI (25 kDa) at a N/P ratio (nitrogen of PEI/phosphate of DNA) of 10:1 unless otherwise mentioned. After 4 hours of transfection, the medium was replaced with fresh 10% serum medium. The transfected cells were further incubated in hypoxic (1% oxygen, 5% CO_2, 95% nitrogen) and normoxic (20% oxygen, 5% CO_2, 75% nitrogen) conditions for 24 hours. It has been known that erythropoietin enhancer/SV promoter-containing plasmid vectors increase target gene expression 6 hours after exposure to hypoxia and the expression levels are rapidly recovered within 1 hour after eliminating hypoxic conditions.[46] For measurement of luciferase activity, treated cells were lysed using reporter lysis buffer (Luciferase Assay Kit, Promega). The luciferase activity in cell lysates was monitored using a SIRIUS luminometer (Berthold Detection System GmbH, Pforzheim, Germany) with

an integration measurement time of 20 seconds. The relative values of luciferase activity were expressed in terms of relative light units/mg of cell protein. The protein concentration of the extract was determined using a bicinchoninic acid protein assay kit (Pierce, Iselin, NJ). The cytotoxicity assay was carried out in a 24-well plate at a density of 1×10^4 MSCs/well. After performing the transfection process, the cells were assayed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide to assess cell viability. The relative cell viability was calculated and expressed as percentage of viable cells. For VEGF plasmid transfection experiments, the conditioned medium was harvested at desired time intervals. The level of VEGF protein in the medium was measured using a human ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. All of the data are presented as the mean ± standard deviation (SD) of three replicate samples. In order to characterize the phenotype of the isolated MSCs and HI-VEGF-MSCs, cells were analyzed by flow cytometry after staining with fluorescence-labeled monoclonal antibodies for CD34-phycoerythrin, CD45-phycoerythrin, and CD90-fluorescein isothiocyanate (BD Biosciences, San Diego, CA). In brief, the harvested cells (1×10^6 cells/ml) were incubated with antibodies (0.5 mg/ml) for 15 minutes at room temperature and then washed twice with phosphate-buffered saline. Fluorescent intensities of cells labeled with antibodies were detected using fluorescence-activated cell sorting analysis (Becton Dickinson, San Jose, CA).

Myocardial infarction, intramyocardial cell transplantation, and histology. Experimental myocardial infarction was induced in 8-week-old male Sprague–Dawley rats (240 ± 10 g) as described earlier.[43] Under general anesthesia, the rat heart was exposed through a 2 cm incision at the left lateral costal rib. The LAD was ligated with a 6-0 silk suture (Ethicon, Somerville, NJ) for 1 hour, followed by reperfusion. Ischemia was confirmed by visual inspection of blanching in the myocardium distal to the site of occlusion. In sham-operated rats, the same procedure was performed without LAD-ligation. For intramyocardial cell transplantation 1 hour immediately after the surgical occlusion, MSCs, and HI-VEGF-MSCs (2.0×10^6 cells) were injected in a total volume of 60 µl serum-free medium with a 30-gauge needle into the anterior and lateral aspects of the contracting wall bordering the infarct. To prepare HI-VEGF-MSCs, MSCs were transfected using the pEpo-SV-VEGF/LPEI formulations prepared at a N/P ratio of 10:1 and were further incubated for 24 hours prior to implantation. For histological cell tracking, MSCs were labeled with DAPI (1 µg/ml, Molecular Probes, Eugene, OR) for 30 minutes on the day of implantation, according to the manufacturer's suggested protocol.

Four groups of animals were used: Normal (sham operation); Control (LAD-ligation and saline treatment); MSCs (LAD-ligation and MSC injection); and HI-VEGF-MSCs (LAD-ligation and HI-VEGF-MSC injection). To investigate the effects of infarction on *in vivo* VEGF production by HI-VEGF-MSC transplanted into myocardium, noninfarcted rats were also transplanted with HI-VEGF-MSCs using the same method as that used in the infarcted rats. Two weeks after cell transplantation, the animals were killed and used for histological examination. The heart tissues were perfusion-fixed with 10% formaldehyde, embedded in paraffin, and cut into 5 µm sections. Every third section was collected for further analysis. All histological measurements were done in a blinded manner. For assessment of *in vivo* VEGF production following cell implantation, rat hearts, and blood were harvested 48 hours postimplantation. The levels of VEGF in the heart tissue and blood extracts were analyzed by ELISA method according to the directions of the manufacturer. To analyze MSC engraftment within infarcted myocardia, DAPI-stained MSC-treated animals were killed 3 days after implantation. Fluorescence images were scanned by a fluorescence microscope (Olympus IX71, Tokyo, Japan) using the excitation filter G365. For immunohistochemical examination of human VEGF, the deparaffinized tissue sections were incubated with primary monoclonal antibody (antihuman VEGF, 1:100 dilution, AbCam, Cambridge, MA), following incubation in biotinylated pan-specific universal secondary antibody. The samples were further processed by a streptavidin/peroxidase complex method using a Vectastain Universal Quick Kit (Vector Laboratories), followed by 3,3-diaminobenxidine (Vector Laboratories) staining. The nuclei were counterstained with hematoxylin. The images were distinguished at ×100 magnification. For analysis of histological changes, all histology samples were observed within the infarct border zone.

Immunohistochemistry. Staining for CD31, also known as platelet endothelial cell adhesion molecule 1, can provide a partial measure of the microvasculature.[43] For immunohistochemical examination of CD31, tissue sections were deparaffinized and subjected to antigen retrieval by microwave heating for 10 minutes in 10 mmol/l sodium citrate (pH 6.0).

The sections were blocked in 2.5% normal horse serum and incubated with 3% H_2O_2 in methanol to quench endogenous peroxidase activity. The samples were then incubated with primary monoclonal antibody (anti-CD31, at 1:50 dilution, AbCam), followed by incubation with biotinylated pan-specific universal secondary antibody. The sections were then processed by a streptavidin/peroxidase complex method with a Vectastain Universal Quick Kit, followed by 3,3-diaminobenxidine staining. Nuclei were counterstained with methyl green. All images were generated under light microscopy and were processed using MetaMorph software ver 4.6 (Universal Imaging, Downingtown, PA). The numbers of CD31-positive cells per square millimeter were determined by counting whole sections in a blinded fashion at ×100 magnification.

TUNEL assay. TUNEL assays were using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA) on the myocardia, according the manufacturer's instructions. A positive control sample was prepared from a normal heart section treated with DNase I (10 U/ml, 20 minutes at room temperature). Apoptotic nuclei were visualized using 3,3-diaminobenxidine (Vector Laboratories). Apoptotic cells were counted under a light microscope. The specimens were then examined over a total of five HPF (×400 magnification) per slice. The value assigned to each specimen was the mean of these five measurements ($n = 6$ per group).

Fibrosis and infarct size analysis. Heart sections were stained with conventional hematoxylin and eosin and Masson's trichrome for analysis of fibrosis. The fibrotic area was measured with MetaMorph software ver 4.6 and was expressed as a percentage of the total left ventricle. For measurement of myocardial infarct size, hearts were sectioned transaxially and incubated in 1% 2,3,5-triphenyltetrazolium chloride (Sigma, St Louis, MO) for 20 minutes at 37 °C. The tissue sections were then fixed in 10% formalin overnight at 2–8 °C. Heart sections were then photographed with a digital camera. Viable myocardium stained deep red, whereas infarct appeared yellow-white. The areas of normal and infarcted left ventricular myocardium were measured with planimetry using ImageJ software from National Institutes of Health. The infarct size of the left ventricle was calculated as the ratio (%) of cumulative infarct area (infarct tissue) to the entire left ventricle area.

Assessment of cardiac function. Two-dimensional echocardiography was performed at baseline (normal rats) and for all operated rats ($n = 6$ per group) at 2 weeks after LAD ligation by an experienced cardiologist blinded to the identity of the groups. Conscious rats were imaged with a GE Vivid Seven ultrasound machine (GE Medical System, Salt Lake City, UT, http://www.gehealthcare.com) with a 10.0 MHz transducer. Data were analyzed using digitized pictures of the short-axis views.

Statistical analysis. Data are presented as the mean ± SD. Comparisons of two groups were made by Student's *t*-test. A P values <0.05 were considered statistically significant.

ACKNOWLEDGMENTS

This research was supported by grants from the Korea Healthcare Technology R&D Project, *Ministry for Health, Welfare & Family Affairs*,

Republic of Korea (A085136), a faculty research grant of Yonsei University College of Medicine for 2010 (8-2010-0019), and the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (20090081874 and 20100022471).

REFERENCES

1. Nabel, EG (2003). Cardiovascular disease. *N Engl J Med* **349**: 60–72.
2. Robey, TE, Saiget, MK, Reinecke, H and Murry, CE (2008). Systems approaches to preventing transplanted cell death in cardiac repair. *J Mol Cell Cardiol* **45**: 567–581.
3. Tae, SK, Lee, SH, Park, JS and Im, GI (2006). Mesenchymal stem cells for tissue engineering and regenerative medicine. *Biomed Mater* **1**: 63–71.
4. Pittenger, MF and Martin, BJ (2004). Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res* **95**: 9–20.
5. Shake, JG, Gruber, PJ, Baumgartner, WA, Senechal, G, Meyers, J, Redmond, JM *et al.* (2002). Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. *Ann Thorac Surg* **73**: 1919–1925; discussion 1926.
6. Tang, YL, Zhao, Q, Zhang, YC, Cheng, L, Liu, M, Shi, J *et al.* (2004). Autologous mesenchymal stem cell transplantation induce VEGF and neovascularization in ischemic myocardium. *Regul Pept* **117**: 3–10.
7. Saito, T, Kuang, JQ, Bittira, B, Al-Khaldi, A and Chiu, RC (2002). Xenotransplant cardiac chimera: immune tolerance of adult stem cells. *Ann Thorac Surg* **74**: 19–24; discussion 24.
8. Cheng, Z, Ou, L, Zhou, X, Li, F, Jia, X, Zhang, Y *et al.* (2008). Targeted migration of mesenchymal stem cells modified with CXCR4 gene to infarcted myocardium improves cardiac performance. *Mol Ther* **16**: 571–579.
9. Noiseux, N, Gnecchi, M, Lopez-Ilasaca, M, Zhang, L, Solomon, SD, Deb, A *et al.* (2006). Mesenchymal stem cells overexpressing Akt dramatically repair infarcted myocardium and improve cardiac function despite infrequent cellular fusion or differentiation. *Mol Ther* **14**: 840–850.
10. Borden, BA, Yockman, J and Kim, SW (2010). Thermoresponsive hydrogel as a delivery scaffold for transfected rat mesenchymal stem cells. *Mol Pharm* **7**: 963–968.
11. Mosca, JD, Hendricks, JK, Buyaner, D, Davis-Sproul, J, Chuang, LC, Majumdar, MK *et al.* (2000). Mesenchymal stem cells as vehicles for gene delivery. *Clin Orthop Relat Res* (379 Suppl): S71–S90.
12. Ylä-Herttuala, S and Alitalo, K (2003). Gene transfer as a tool to induce therapeutic vascular growth. *Nat Med* **9**: 694–701.
13. Dulak, J, Zagorska, A, Wegiel, B, Loboda, A and Jozkowicz, A (2006). New strategies for cardiovascular gene therapy: regulatable pre-emptive expression of pro-angiogenic and antioxidant genes. *Cell Biochem Biophys* **44**: 31–42.
14. Pons, J, Huang, Y, Arakawa-Hoyt, J, Washko, D, Takagawa, J, Ye, J *et al.* (2008). VEGF improves survival of mesenchymal stem cells in infarcted hearts. *Biochem Biophys Res Commun* **376**: 419–422.
15. Gao, F, He, T, Wang, H, Yu, S, Yi, D, Liu, W *et al.* (2007). A promising strategy for the treatment of ischemic heart disease: Mesenchymal stem cell-mediated vascular endothelial growth factor gene transfer in rats. *Can J Cardiol* **23**: 891–898.
16. Lee, M, Bikram, M, Oh, S, Bull, DA and Kim, SW (2004). Sp1-dependent regulation of the RTP801 promoter and its application to hypoxia-inducible VEGF plasmid for ischemic disease. *Pharm Res* **21**: 736–741.
17. Lee, M, Choi, D, Choi, MJ, Jeong, JH, Kim, WJ, Oh, S *et al.* (2006). Hypoxia-inducible gene expression system using the erythropoietin enhancer and 3′-untranslated region for the VEGF gene therapy. *J Control Release* **115**: 113–119.
18. Lee, M, Rentz, J, Bikram, M, Han, S, Bull, DA and Kim, SW (2003). Hypoxia-inducible VEGF gene delivery to ischemic myocardium using water-soluble lipopolymer. *Gene Ther* **10**: 1535–1542.
19. Yockman, JW, Choi, D, Whitten, MG, Chang, CW, Kastenmeier, A, Erickson, H *et al.* (2009). Polymeric gene delivery of ischemia-inducible VEGF significantly attenuates infarct size and apoptosis following myocardial infarct. *Gene Ther* **16**: 127–135.
20. Haleem-Smith, H, Derfoul, A, Okafor, C, Tuli, R, Olsen, D, Hall, DJ *et al.* (2005). Optimization of high-efficiency transfection of adult human mesenchymal stem cells *in vitro*. *Mol Biotechnol* **30**: 9–20.
21. Otani, K, Yamahara, K, Ohnishi, S, Obata, H, Kitamura, S and Nagaya, N (2009). Nonviral delivery of siRNA into mesenchymal stem cells by a combination of ultrasound and microbubbles. *J Control Release* **133**: 146–153.
22. Cavazzana-Calvo, M, Lagresle, C, Hacein-Bey-Abina, S and Fischer, A (2005). Gene therapy for severe combined immunodeficiency. *Annu Rev Med* **56**: 585–602.
23. Gao, X, Kim, KS and Liu, D (2007). Nonviral gene delivery: what we know and what is next. *AAPS J* **9**: E92–104.
24. Kim, H, Kim, HA, Bae, YM, Choi, JS and Lee, M (2009). Dexamethasone-conjugated polyethylenimine as an efficient gene carrier with an anti-apoptotic effect to cardiomyocytes. *J Gene Med* **11**: 515–522.
25. Hoffmann, J, Glassford, AJ, Doyle, TC, Robbins, RC, Schrepfer, S and Pelletier, MP (2010). Angiogenic effects despite limited cell survival of bone marrow-derived mesenchymal stem cells under ischemia. *Thorac Cardiovasc Surg* **58**: 136–142.
26. Guo, Z, Yang, NS, Jiao, S, Sun, J, Cheng, L, Wolff, JA *et al.* (1996). Efficient and sustained transgene expression in mature rat oligodendrocytes in primary culture. *J Neurosci Res* **43**: 32–41.
27. Toma, C, Pittenger, MF, Cahill, KS, Byrne, BJ and Kessler, PD (2002). Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* **105**: 93–98.
28. Dvorak, HF, Brown, LF, Detmar, M and Dvorak, AM (1995). Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* **146**: 1029–1039.
29. Silva, GV, Litovsky, S, Assad, JA, Sousa, AL, Martin, BJ, Vela, D *et al.* (2005). Mesenchymal stem cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a canine chronic ischemia model. *Circulation* **111**: 150–156.
30. Mangi, AA, Noiseux, N, Kong, D, He, H, Rezvani, M, Ingwall, JS *et al.* (2003). Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med* **9**: 1195–1201.
31. Sadat, S, Gehmert, S, Song, YH, Yen, Y, Bai, X, Gaiser, S *et al.* (2007). The cardioprotective effect of mesenchymal stem cells is mediated by IGF-I and VEGF. *Biochem Biophys Res Commun* **363**: 674–679.
32. Maltepe, E and Saugstad, OD (2009). Oxygen in health and disease: regulation of oxygen homeostasis–clinical implications. *Pediatr Res* **65**: 261–268.
33. Riley, PR and Smart, N (2009). Thymosin beta4 induces epicardium-derived neovascularization in the adult heart. *Biochem Soc Trans* **37**(Pt 6): 1218–1220.
34. Deasy, BM, Feduska, JM, Payne, TR, Li, Y, Ambrosio, F and Huard, J (2009). Effect of VEGF on the regenerative capacity of muscle stem cells in dystrophic skeletal muscle. *Mol Ther* **17**: 1788–1798.
35. Levenberg, S, Rouwkema, J, Macdonald, M, Garfein, ES, Kohane, DS, Darland, DC *et al.* (2005). Engineering vascularized skeletal muscle tissue. *Nat Biotechnol* **23**: 879–884.
36. Tang, YL, Zhao, Q, Qin, X, Shen, L, Cheng, L, Ge, J *et al.* (2005). Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. *Ann Thorac Surg* **80**: 229–236; discussion 236.
37. Gnecchi, M, He, H, Noiseux, N, Liang, OD, Zhang, L, Morello, F *et al.* (2006). Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *FASEB J* **20**: 661–669.
38. Oshima, H, Payne, TR, Urish, KL, Sakai, T, Ling, Y, Gharaibeh, B *et al.* (2005). Differential myocardial infarct repair with muscle stem cells compared to myoblasts. *Mol Ther* **12**: 1130–1141.
39. Germani, A, Di Carlo, A, Mangoni, A, Straino, S, Giacinti, C, Turrini, P *et al.* (2003). Vascular endothelial growth factor modulates skeletal myoblast function. *Am J Pathol* **163**: 1417–1428.
40. Rissanen, TT and Ylä-Herttuala, S (2007). Current status of cardiovascular gene therapy. *Mol Ther* **15**: 1233–1247.
41. Thiel, C and Nix, M (2006). Efficient transfection of primary cells relevant for cardiovascular research by nucleofection. *Methods Mol Med* **129**: 255–266.
42. Song, H, Chang, W, Lim, S, Seo, HS, Shim, CY, Park, S *et al.* (2007). Tissue transglutaminase is essential for integrin-mediated survival of bone marrow-derived mesenchymal stem cells. *Stem Cells* **25**: 1431–1438.
43. Chang, W, Song, BW, Lim, S, Song, H, Shim, CY, Cha, MJ *et al.* (2009). Mesenchymal stem cells pretreated with delivered Hph-1-Hsp70 protein are protected from hypoxia-mediated cell death and rescue heart functions from myocardial injury. *Stem Cells* **27**: 2283–2292.
44. Lee, M, Rentz, J, Han, SO, Bull, DA and Kim, SW (2003). Water-soluble lipopolymer as an efficient carrier for gene delivery to myocardium. *Gene Ther* **10**: 585–593.
45. Jiang, B, Dong, H, Zhang, Z, Wang, W, Zhang, Y and Xu, X (2007). Hypoxic response elements control expression of human vascular endothelial growth factor(165) genes transferred to ischemia myocardium *in vivo* and *in vitro*. *J Gene Med* **9**: 788–796.
46. Shibata, T, Akiyama, N, Noda, M, Sasai, K and Hiraoka, M (1998). Enhancement of gene expression under hypoxic conditions using fragments of the human vascular endothelial growth factor and the erythropoietin genes. *Int J Radiat Oncol Biol Phys* **42**: 913–916.