Original Research

Novel vascular endothelial growth factor gene delivery system-manipulated mesenchymal stem cells repair infarcted myocardium

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Abstract

Transplantation of vascular endothelial growth factor (VEGF) gene-manipulated mesenchymal stem cells (MSCs) has been proposed as a promising therapy strategy for cardiac repair after myocardium infarction. However, the gene delivery system, including targeted VEGF gene and delivery vehicle, still needs to be optimized. In this study, a novel, hyperbranched poly(amidoamine) (hPAMAM), polymer-based, hypoxia-regulated $VEGF_{165}$ plasmid (pHRE-$VEGF_{165}$) delivery system was constructed for effective, biocompatible and controllable gene expression. The hPAMAM demonstrated high transfection efficiency ($38.98 \pm 1.95\%$) with minor cytotoxicity (cell viability $= 92.38 \pm 1.09\%$) in primary MSCs under optimal conditions. Under hypoxia, hPAMAM-pHRE-h$VEGF_{165}$-transfected MSCs could over-express h$VEGF_{165}$ stably for 14 days, with a peak expression at day 2, which promoted endothelial cell proliferation *in vitro*. The transplantation of hPAMAM-pHRE-h$VEGF_{165}$ gene delivery system-manipulated MSCs could enhance ischemic myocardium VEGF concentration obviously, which improved the graft MSC survival, increased neovascularization, and ultimately preserved cardiac function to a significantly greater degree than untreated MSC transplantation. This work demonstrated that hPAMAM-based pHRE-h$VEGF_{165}$ gene delivery combined with MSC transplantation is an economical, feasible and biocompatible strategy for cardiac repair.

Keywords: hyperbranched poly(amidoamine), mesenchymal stem cell, vascular endothelial growth factor, cardiac repair

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Introduction

Mesenchymal stem cell (MSC) transplantation has received growing attention as a promising treatment for myocardial infarction.[1] A more efficient approach will be the transplantation of exogenous angiogenic gene-manipulated MSCs in ischemic myocardium. This combined cellular and gene treatment strategy has shown great efficacy for cardiac repair.[2,3] However, the gene delivery system, including angiogenic gene and delivery vehicle, still needs to be optimized.

The exogenous angiogenic gene could stimulate neovasculaturization in ischemic tissue.[4] More than 20 growth factors have been identified with angiogenic activities, among which vascular endothelial growth factor (VEGF) is the major regulator of blood vessel formation, both during development and in adulthood. VEGF has been widely studied both in animal and human studies. $VEGF_{165}$ is the predominant isoform, with strong angiogenic potency.[5] Cellular angiogenesis using genetically engineered MSCs has more advantages, such as localized transgene expression and reduced inflammation. However, long-term over-expression of VEGF *in vivo* has been observed to lead to hemangioma formation instead of functional vessels in animal models.[6–9] Therefore, the ideal VEGF controllable system is to let the gene expression respond to hypoxia. VEGF expression should be turned off when the blood supply to the myocardium is adequate. Therefore, the controllable VEGF expression *in vivo* is necessary for clinical application.

As we all know, hypoxia-inducible factor-1 alpha subunit (HIF-1α) is one of the key mammalian transcription factors that exhibit dramatic increases in both protein stability and intrinsic transcriptional potency during low-oxygen stress.[10] Hypoxia response element is a DNA fragment which could bind HIF-1α at the transcriptional level and launch the gene expression.[11] We hypothesized that the hypoxia response

element could be inserted into the promoter region of the human $VEGF_{165}$ ($hVEGF_{165}$) gene to form hypoxia-regulated $hVEGF_{165}$ plasmids (pHRE-$hVEGF_{165}$) for controllable gene expression. The hypoxia condition caused by ischemic myocardium could up-regulate VEGF expression. When the regional oxygen supply is adequate, VEGF expression could be turned off by the normoxia condition. Hence, the hypoxia response element-modified VEGF gene is a good candidate for controllable VEGF expression in ischemic myocardium.

The most demanding task in gene delivery systems is the development of efficient and non-toxic gene carriers. Viral vehicles, including adenovirus, adeno-associated virus and retrovirus, have high gene transfection efficiency.[12] However, they have limited clinical application due to their inherent potential for immunogenicity, tumorigenicity and induction of an inflammatory response.[13] It is therefore necessary to develop a safer and more efficient non-viral gene carrier that can circumvent the limitations of viral vehicles. Polymer gene vehicles have several advantages compared with viral vectors, including safety, stability, and large plasmid DNA-loading capacity.[14] By condensing plasmid DNA into nanoparticles through electrostatic interactions, polymeric vehicles can protect DNA from nuclease degradation, facilitate endosome–lysosome escape and induce cellular uptake and gene expression.[15] However, the drawbacks of polymer vehicles, such as complex synthesis procedure, limited transfection efficiency and high cytotoxicity, still need to be overcome.[16]

Poly(amidoamine) (PAMAM) dendrimer is a promising polymeric gene vehicle with minor cytotoxicity and encouraging transfection efficiency.[17] Some recent studies have further modified the dendrimer surface using polyethylene glycol (PEG), β-cyclodextrins and L-arginine to improve the transfection efficiency and reduce the cytotoxicity further.[18] However, the synthetic procedure of PAMAM dendrimer itself and surface modification is rather complex, which limit its large-scale production and application.[19] Previous studies reported that hyperbranched PAMAM (hPAMAM) was structurally analogous to dendrimers and had similar architecture and properties in terms of globular topology, low viscosity, high solubility and high density of functional groups, whereas they can be synthesized by a simpler and more economical one-step/pot polymerization technique.[20] In an earlier study, our co-workers optimized the synthesis procedure of hPAMAM.[21] To date, no studies have reported that hPAMAM could be applied to deliver targeted genes into primary stem cells. In the present study, we optimized the delivery of the hPAMAM-based pHRE-$hVEGF_{165}$ gene into primary MSCs, and grafted the genetically manipulated MSCs in myocardial infarction models. We anticipate that this novel gene delivery system combined with MSC-mediated cellular therapy will enable a new approach for cardiac repair.

Materials and methods

Synthesis of hPAMAM

With diethylene triamine (DETA) and methyl acrylate (MA) as raw materials, hPAMAM nanoparticles were synthesized by repeated Michael addition and amidation.[21] Briefly, DETA (20.6 g) was dissolved in methanol (25 mL) and MA (20.6 g) was added dropwise into the reaction system. At room temperature, the reaction system was stirred for 48 h. Then, the methanol was removed by a rotary evaporator under a vacuum. The reaction continued for one hour at 60°C, one hour at 80°C, 1.5 h at 100°C, 1.5 h at 120°C and three hours at 140°C to achieve hPAMAM. After precipitation in ethyl oxide three times, the resulting product was kept in a sealed container for the following experiments.

Combination of hPAMAM and DNA

Enhanced green fluorescent protein plasmids (pEGFP) (4.7 kb) were purchased from Gene Chem (Shanghai, China). The $hVEGF_{165}$ gene was cloned into the pGMT-easy plasmid (Promega, Madison, WI, USA). Then cDNA containing the $hVEGF_{165}$ gene was subcloned into the pGL-3-promoter vector (Promega) to replace the luciferase gene. Five copies of hypoxia response element (Gene Chem) were inserted into the pGL-3 plasmid upstream of the SV40 promoter to construct pHRE-$hVEGF_{165}$ (4.25 kb).

The hPAMAM nanoparticles and 2 μg DNA (pEGFP or pHRE-$hVEGF_{165}$) were diluted separately in 50 μL of 150 mmol/L NaCl. The diluted DNA was then added to the nanoparticle solution at different weight ratios ($w_{\text{h-PAMAM}}/w_{\text{DNA}} = 2$, 4, 6, 8, 10, 12 and 14) to form hPAMAM–DNA complexes.

Isolation and culture of MSCs

All animals were cared for and maintained at the Animal Center, Zhongshan Hospital, Fudan University, and were approved by the Institutional Review Board and Institutional Animal Care and Use Committee Protocols of Fudan University. The syngenic male and female mouse strain C57/BL6 weighing 20–25 g was used to avoid rejection.

Bone marrow was flushed from tibias and femurs of male donor mice using a 25-gauge needle. Whole marrow cells were cultured at $1\times10^6/\text{cm}^2$ in MesenCult basal medium that was supplemented with MSC stimulatory supplements (Cyagen Biosciences Inc., Goleta, CA, USA). The non-adherent cells were removed by a medium change at 36 and 72 h. The medium was exchanged every three days throughout the study. All experiments were carried out with cells passaged no more than five times.

Characterizations of MSCs

The characterizations of MSCs were evaluated according to their surface markers, adherent ability and pluripotency differentiation assay.

The defined cell surface markers (CD29, CD34, CD44 and Sca-1) and required negatively expressed marker (CD117) on cultured MSCs were evaluated using a flow cytometer (FACSAriaII; BD, Franklin Lakes, NJ, USA).

MSCs were seeded as a monolayer in six-well plates. OriCellTM C57BL/6 mouse MSC adipogenic and osteogenic

differentiation kits (Catalog no. MUBMX-90031 and MUBMX-90021) were used according to the manufacturer's instructions (Cyagen Biosciences Inc.). When the adipogenic and osteogenic differentiation processes were completed, the cells were fixed with 4% paraformaldehyde for 30 min, stained with Oil Red O working solution and alizarin red working solution, respectively, and then rinsed with phosphate-buffered saline (PBS). The pictures were taken under a light microscope (Olympus, Tokyo, Japan).

Optimization of gene transfection

Before the transfection experiments, adherent cells were rinsed with PBS, trypsinized and seeded in six-well plates at a density of 1×10^5 cells/well with complete medium without antibiotics. After 24 h, the culture medium was replaced by Opti-MEM medium (Gibco Invitrogen, Carlsbad, CA, USA). The hPAMAM-pEGFP complexes at various weight ratios were then added into the plates. Four hours later, the transfection medium was replaced by fresh MesenCult basal medium containing MSC stimulatory supplements. After 24 h, the pEGFP-transfected MSCs were observed under a fluorescence microscope (Leica DM IRE2; Leica, Solms, Germany). To confirm the optimal transfection conditions, the transfection efficiency at various weight ratios were evaluated by a flow cytometer. Mock-transfected cells were used for setting the autofluorescence baseline. Lipofectamine 2000 and polyetherimide (PEI; MW = 25 kDa) (Sigma-Aldrich, St Louis, MO, USA) were used to deliver pEGFP into MSCs as controls. Data were analyzed using Flowjo software (version 7.6; Treestar, Ashland, OR, USA) with gating at 1% for the optimal $w_{\mathrm{hPAMAM}}/w_{\mathrm{DNA}}$ ratio during transfection. To determine the optimal DNA dosage for transfection, 1, 3, 4 and 5 μg DNA were transfected into cells using the above process simultaneously.

Cytotoxicity of hPAMAM

hPAMAM–DNA complexes were transfected into MSCs at various weight ratios (2, 4, 6, 8, 10, 12 and 14) and DNA dosages (1, 2, 3, 4 and 5 μg) in the same manner as described above. After 24 h, MSCs were stained using an Annexin V-PE apoptosis detection kit (Beyotime, Haimen, China). The dead and apoptotic cells could be identified and analyzed by flow cytometry. The cell viability was calculated according to the following equation: Cell viability = $1-(D+A)$, where D is the percentage of dead cells and A is the percentage of aopototic cells.

In vitro hVEGF$_{165}$ expression

The culture media were pre-equilibrated with a hypoxic gas mixture (5% O_2, 5% CO_2 and 94% N_2). The hPAMAM-pHRE-hVEGF$_{165}$ complexes-transfected MSCs were replenished with the hypoxic media, placed in the hypoxia incubator (5% O_2, 1% CO_2 and 94% N_2) and maintained at 37°C. The gene expression efficiency was analyzed as follows.

One day after transfection, samples were immunostained using anti-hVEGF$_{165}$ primary antibody (R&D, Santa Fe Springs, CA, USA), and 4,6-diamidino-2-phenylindole was used for nuclear counterstaining. The immunostained cells were visualized using a confocal microscope (Olympus).

At 2, 4, 8 and 14 days after transfection, transfected cell samples were collected to quantify hVEGF$_{165}$ gene expression using realtime polymerase chain reaction (real-time PCR). The non-transfected MSCs under hypoxia and normoxia, and transfected cells under normoxia were used as controls. The primers for hVEGF$_{165}$ amplification were (forward) 5′ ATGAACTTTCTGCTGTCTTGGGTG 3′; (reverse) 5′ TCACCGCCTCGGCTTGTCACA 3′; and 18 s expression was used as the internal control. After isolation of total RNA and cDNA synthesis, the PCR thermal cycling program was: 1 cycle of enzyme activation at 95°C for 30 s, then annealing at 58°C for 30 s and extension at 72°C for 30 s for 40 cycles.

At regular two-day intervals (from day 0 until day 14 after transfection), the culture medium of the transfected MSCs was collected and kept frozen at −70°C until use. The non-transfected MSCs under hypoxia and normoxia, and transfected cells under normoxia were used as controls. Enzyme-linked immunosorbent assay (ELISA) was performed according to the instructions from the supplier (R&D).

In vitro endothelial cell proliferation assay

To determine the angiogenic proliferation induced by the transfected MSCs, *in vitro* time-course endothelial cell proliferation assays were performed. Human umbilical vein endothelial cells (HUVECs), which were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), were seeded in 96-well plates at a density of 5×10^3 cells/well. Under the hypoxia condition, MSCs were seeded in six-well plates and then transfected using the optimal weight ratio and DNA quantity. From day 0 (pre-transfection) to day 5 (post-transfection) after transfection, 100 μL of culture medium from the transfected MSCs was collected and transferred to HUVECs everyday. HUVEC proliferation was measured by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide blue-indicator dye]-based (MTT) assay on day 0, 1, 3 and 5. Briefly, MTT solution (20 μL, 5 mg/mL in PBS) was added into each well and incubated for four hours. The medium was then removed and 150 μL dimethyl sulfoxide was added to dissolve the formazan crystal formed by viable cells. The absorption was measured at 490 nm using a microplate reader (Model 680; Bio-Rad Laboratories; Hercules, CA, USA). Culture medium from the non-transfected MSCs under hypoxia and transfected ones under normoxia were used to treat HUVECs as controls. HUVEC proliferation at day 0 was normalized at 100%.

Myocardial infarction models and MSC transplantation

Female C57/BL6 mice were anesthetized under 4% isoflurane for induction, intubated and kept under 1% isoflurane for maintenance of anesthesia. The myocardial infarction

models were created by permanently ligating the left anterior descending coronary artery with a 8-0 polypropylene snare, 2 mm distal to the left auricle. After confirming successful ligation of the left anterior descending coronary artery by observation of visible blanching of the left ventricle, 50 μL of DMEM without MSCs (Group 1), or with 1×10^6 male MSCs (Group 2), or with 1×10^6 hPAMAM-pHRE-hVEGF$_{165}$-transfected male MSCs (Group 3) were intramyocardially injected at the border zone of the infarct. To assess the gene expression efficiency of this system under normal conditions *in vivo*, further animals without myocardial infarction received 1×10^6 hPAMAM-pHRE-hVEGF$_{165}$-transfected male MSCs (Group 4).

VEGF concentration in myocardium

To verify the concentration of VEGF in the heart following injection of VEGF-transfected myoblasts into the myocardium, VEGF concentrations were measured three days after surgery using a commercially available hVEGF$_{165}$ ELISA kit. The hearts of four groups ($n = 5$ in each group) were weighed, and homogenized in 1× TE buffer containing protease inhibitors. Hundred microliters of the supernatant was used for determination of VEGF concentrations by ELISA assay.

Survival of grafted MSCs

The sex-determining region of the Y-chromosome (sry) gene copy number was used to quantify the survival of male grafted MSCs on realtime PCR. The DNA from homogenized heart tissue was extracted by using the DNeasy kit (QIAGEN, Dusseldorf, Germany), according to the manufacturer's instructions. The Sry gene was extracted from male MSCs and amplified in *Escherichia coli*. Sry plasmids were then extracted and sequenced. Standard curves were generated by serially diluting the Sry plasmids.[22] Realtime PCR analysis was performed using the SYBR Green kit (QIAGEN). The primers were as follows: (forward) 5′ CGTCGGAAGGCGAAGATG 3′; (reverse) 5′ GCGTTGAT GGGCGGTAAGT 3′. The Sry gene copy number in Groups 2, 3 and 4 was determined at 10 min on days 1, 3 and 7 ($n = 5$ for each time-point in each group) after gender-mismatch transplantation.

Heart function assessment

At four weeks after cell transplantation, heart function ($n = 8$ in each group) was measured by an investigator blinded to the therapeutic intervention on the animals using an echocardiograph (Vevo770; VisualSonics, Toronto, Canada).

From an M-mode echocardiogram, measurements were obtained for left ventricular anterior wall thickness at end-diastole (LVAWTed) and end-systole (LVAWTes), and left ventricular internal diameter at end-diastole (LVIDed) and end-systole (LVIDes). Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were computed using the following formulae: LVEF $= 1 -$ (LVIDes/LVIDed)^2 and LVFS $= 1 -$ (LVIDes/LVIDed).

Immunohistochemistry for angiogenesis

The day after heart function assessment, mice were sacrificed by overdose of isoflurane gas inhalation. The hearts were harvested, perfused with heparin–PBS and fixed in 10% formalin at 4°C till sectioning. Hearts were sectioned into five segments parallel to the apex–base axis. Immunohistochemical staining was performed on the sections of formalin-fixed, paraffin-embedded tissue. Sections were air-dried at room temperature and then placed in a 60°C oven for 30 min to melt the paraffin.

The capillary densities at the border zone were measured by staining for PECAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) ($n = 5$ in each group). When counting vessel numbers, five sections of the peri-infarct area were chosen in each mouse. For each section, five fields were evaluated at high magnification.

Statistical analysis

The data were analyzed with SPSS 17.0 software (SPSS, Chicago, IL, USA). All values were expressed as the mean $\pm$ standard error of the mean. One-way analysis of variance with the *post hoc* Bonferroni test was performed to assess the significant difference among multiple groups. The significant difference between two groups was evaluated using Student's *t*-test.

Results

Characterizations of primary MSCs

The positive expression of defined surface markers was 99.93% for CD29, 91.67% for CD34, 89.57% for CD44 and 98.33% for Sca-1. The expression of CD117, which is the required negatively expressed marker, was 1.29% of isolated MSC cultures (Figure 1a). The cultured MSCs readily adhered to plastic culture dishes and formed fibroblast-like colonies in a few days (Figure 1b). The adipogenic and osteogenic differentiation assay of the isolated MSCs is shown in Figure 1c.

Optimization of transfection protocol

When the hPAMAM-DNA complexes were transfected into MSCs at various weight ratios and DNA quantities, maximum transfection efficiency ($38.98 \pm 1.95\%$) could be achieved when the $w_{\mathrm{hPAMAM}}/w_{\mathrm{DNA}}$ ratio was 10 using 2μg DNA per 1×10^5 cells. The hPAMAM showed a much higher transfection efficiency than Lipofectamine 2000 ($31.44 \pm 0.85\%$, $P < 0.05$) and PEI ($22.69 \pm 1.73\%$, $P < 0.01$) in primary MSCs (Figure 2a). Changing DNA dosage did not enhance transfection efficiency significantly (Figure 2b). Therefore, the optimal transfection condition of hPAMAM was a $w_{\mathrm{hPAMAM}}/w_{\mathrm{DNA}}$ ratio of 8 using 2 μg DNA per 1×10^5 cells. The transfected MSCs under optimal condition are shown in Figure 2c.

hPAMAM showed minor cytotoxicity at 24 h after transfection. Under the optimal transfection condition ($w_{\mathrm{hPAMAM}}/w_{\mathrm{DNA}}$ ratio = 8), the cell viability was $92.38 \pm 1.09\%$. Even though the weight ratio achieved 14, the cell

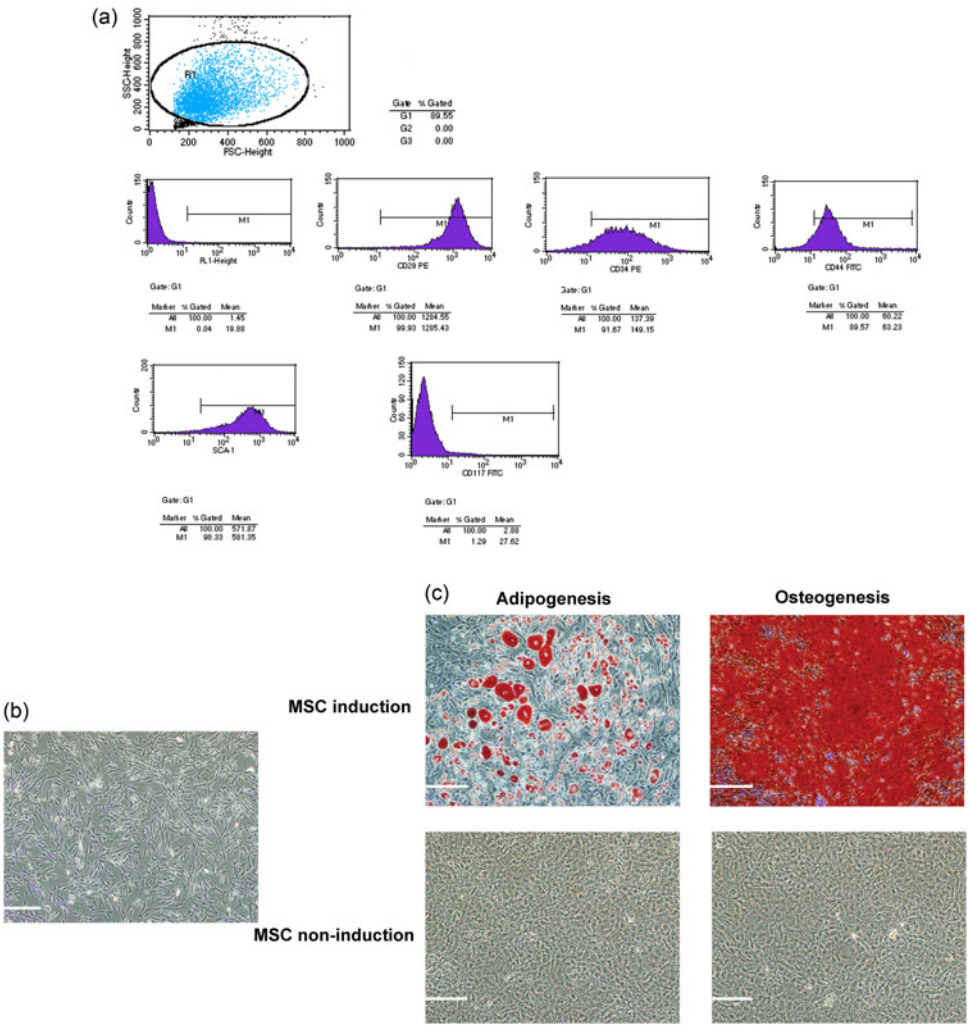

Figure 1 Characterizations of isolated MSCs. (a) Surface marker expression of the isolated MSCs. (b) Proliferation of the isolated MSCs. (c) Differentiation assays show adipogenesis and osteogenesis of MSCs. MSC, mesenchymal stem cell. (A color version of this figure is available in the online journal)

viability was still above 90% (Figure 2d). In addition, the variation of DNA quantity did not increase the cytotoxicity obviously during transfection (Figure 2e).

hPAMAM-pHRE-hVEGF$_{165}$ complexes transfection into MSCs

Immunostaining showed that the hPAMAM-pHRE-hVEGF$_{165}$ complexes-transfected MSCs could express hVEGF$_{165}$ under hypoxia (Figure 3a to c). Using realtime PCR and ELISA assay, the hVEGF$_{165}$ time-course gene expression and protein concentration profile of transfected MSCs could be determined. Under hypoxia, the gene expression increased 8.50 ± 0.60 times at day 2, 6.25 ± 0.99 times at day 4, 4.02 ± 0.25 times at day 8 and 2.88 ± 0.49 times at day 14 compared with that of non-transfected MSCs (Figure 3d). The transfected MSCs could stably secrete hVEGF$_{165}$ protein for 14 days, with a peak expression level (3449.68 ± 283.84 pg/mL) at day 2 after transfection (Figure 3e).

HUVEC proliferation is expected to be stimulated and enhanced by hVEGF$_{165}$ expression from transfected MSCs. As shown in Figure 3f, with three days of incubation, HUVECs treated with culture medium from hPAMAM-hVEGF$_{165}$ complexes-transfected MSCs demonstrated greater proliferation than control cells (HUVECs treated with medium from non-transfected MSCs; $P < 0.01$). With five days of treatment, HUVEC proliferation was significantly accelerated ($P < 0.01$).

Myocardial VEGF concentration and graft MSC survival

The VEGF concentration in Group 1 averaged 11.38 ± 1.18 pg/g heart tissue, while the concentration in Group 2 averaged 48.06 ± 4.36 pg/g heart tissue. Group 3 showed a much higher level of VEGF expression than both Group 1 and 2 (86.59 ± 2.95 pg/g, $P < 0.01$ versus Groups 1 and 2). The VEGF concentration in Group 4 averaged 13.47 ± 1.29 pg/g heart tissue ($P < 0.01$ versus Groups 2 and 3) (Figure 4a). Detection of hVEGF$_{165}$ in Groups 1 and 2 was most likely due to cross-reactivity between hVEGF$_{165}$ and endogenous mice VEGF.

The threshold cycle of the Sry gene in male MSCs has a good relation with serially diluted cells (Figure 4b and c),

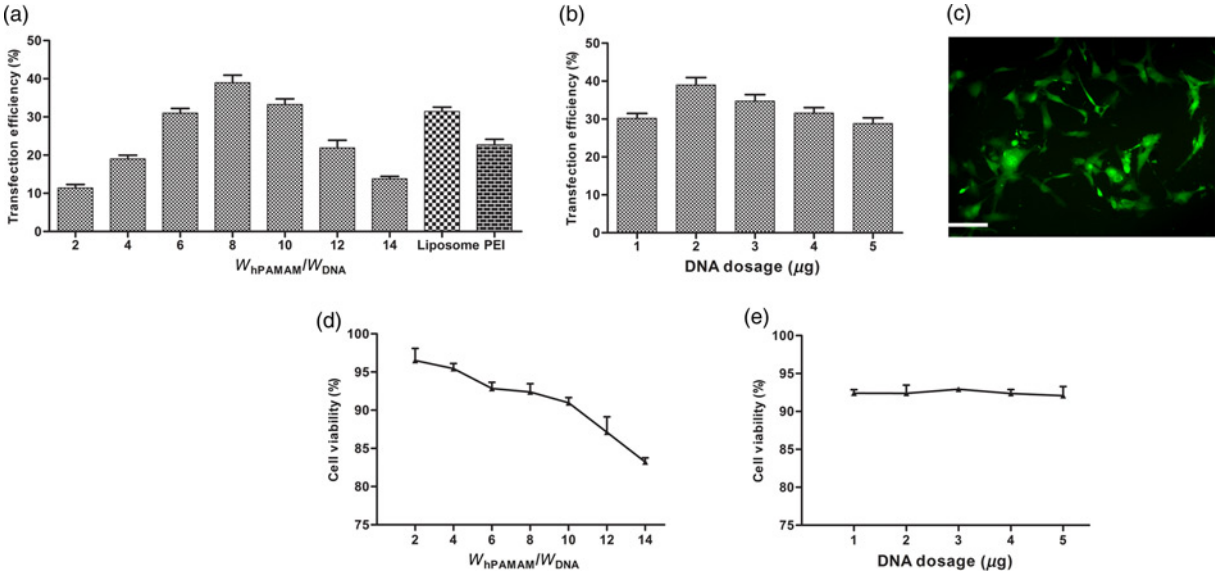

Figure 2 Optimization of hPAMAM-based gene transfection. (a) Transfection efficiency of hPAMAM at various w_{hPAMAM}/w_{DNA} ratios. (b) Transfection efficiency of hPAMAM at various DNA dosages. (c) hPAMAM-pEGFP-transfected MSCs under optimal condition. (d) Cytotoxicity of hPAMAM at various w_{hPAMAM}/w_{DNA} ratios. (e) Cytotoxicity of hPAMAM at various DNA dosages. Scale bar = 50 μm (c). hPAMAM, hyperbranched poly(amidoamine); pEGFP, enhanced green fluorescent protein plasmid; MSC, mesenchymal stem cell. (A color version of this figure is available in the online journal)

R^2= 0.9848. Realtime PCR assay of the Sry gene quantified the number of surviving male MSCs in female hearts of Groups 2, 3 and 4. The survival ratio was calculated by the survival MSC number to the total grafted cells. The survival rate was assayed four hours, 24 h, and seven days after transplant, as shown in Figure 4d. Groups 3 and 4 demonstrated significantly higher MSC survival rates than Group 2 (4 h: 41.12 $\pm$ 1.59% and 48.11 $\pm$ 2.34% versus 17.52 $\pm$ 2.31%, $P < 0.01$; 24 h: 25.37 $\pm$ 2.42% and 29.83 $\pm$ 1.44% versus 8.12 $\pm$ 0.69%, $P < 0.01$; 7 d: 13.50 $\pm$ 0.81% and 18.56 $\pm$ 1.11 versus 5.89 $\pm$ 1.20%, $P < 0.01$).

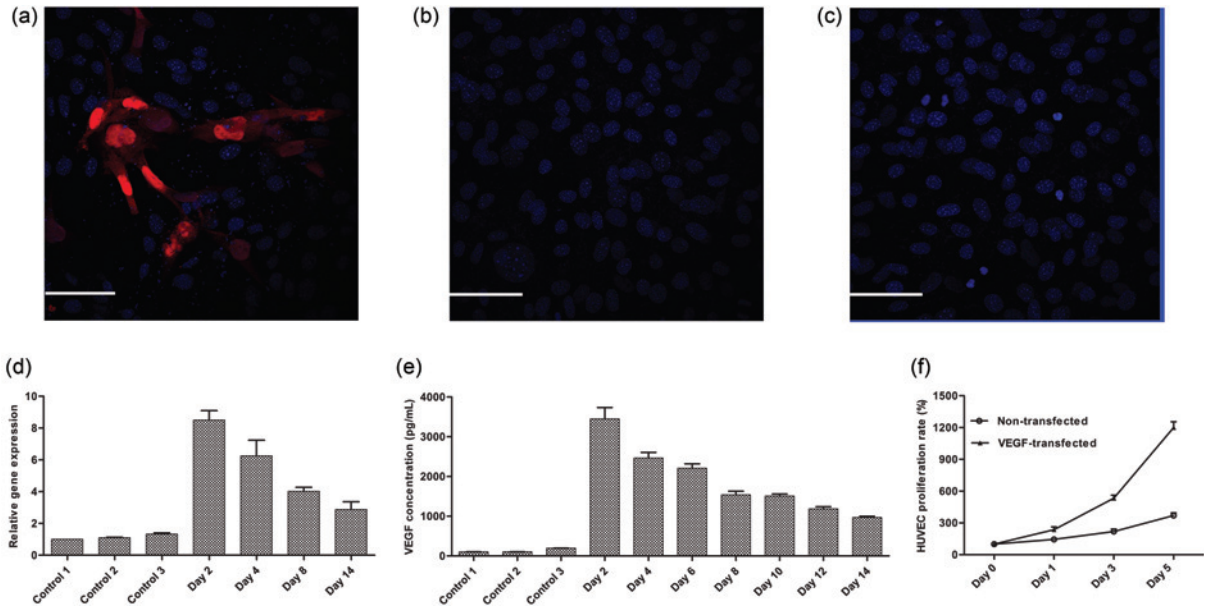

Figure 3 hPAMAM-based pHRE-hVEGF$_{165}$ transfection and HUVEC proliferation assay. (a) hVEGF$_{165}$ expression (red) from hPAMAM-pHRE-hVEGF$_{165}$-transfected MSCs under hypoxia. Nuclei were counterstained with DAPI (blue). (b) hPAMAM-pHRE-hVEGF$_{165}$-transfected MSCs under normoxia. (c) Non-transfected MSCs under hypoxia. (d) hVEGF$_{165}$ gene expression of hPAMAM-pHRE-hVEGF$_{165}$-transfected MSCs under hypoxia during 14 days after transfection (Control 1: non-transfected MSCs under hypoxia; Control 2: non-transfected MSCs under normoxia; Control 3: transfected MSCs under normoxia). (e) hVEGF$_{165}$ protein expression of hPAMAM-pHRE-hVEGF$_{165}$-transfected MSCs under hypoxia during 14 days after transfection (Control 1: non-transfected MSCs under hypoxia; Control 2: non-transfected MSCs under normoxia; Control 3: transfected MSCs under normoxia). (f) The proliferation assay of HUVECs treated with culture medium from hPAMAM-pHRE-hVEGF$_{165}$ complexes-transfected MSCs and non-transfected MSCs. Scale bar = 50 μm (a, b and c). hPAMAM, hyperbranched poly(amidoamine); pHRE-hVEGF$_{165}$, polymer-based hypoxia-regulated VEGF$_{165}$ plasmid; HUVEC, human umbilical vein endothelial cell; MSC, mesenchymal stem cell; VEGF, vascular endothelial growth; DAPI, 4,6-diamidino-2-phenylindole. (A color version of this figure is available in the online journal)

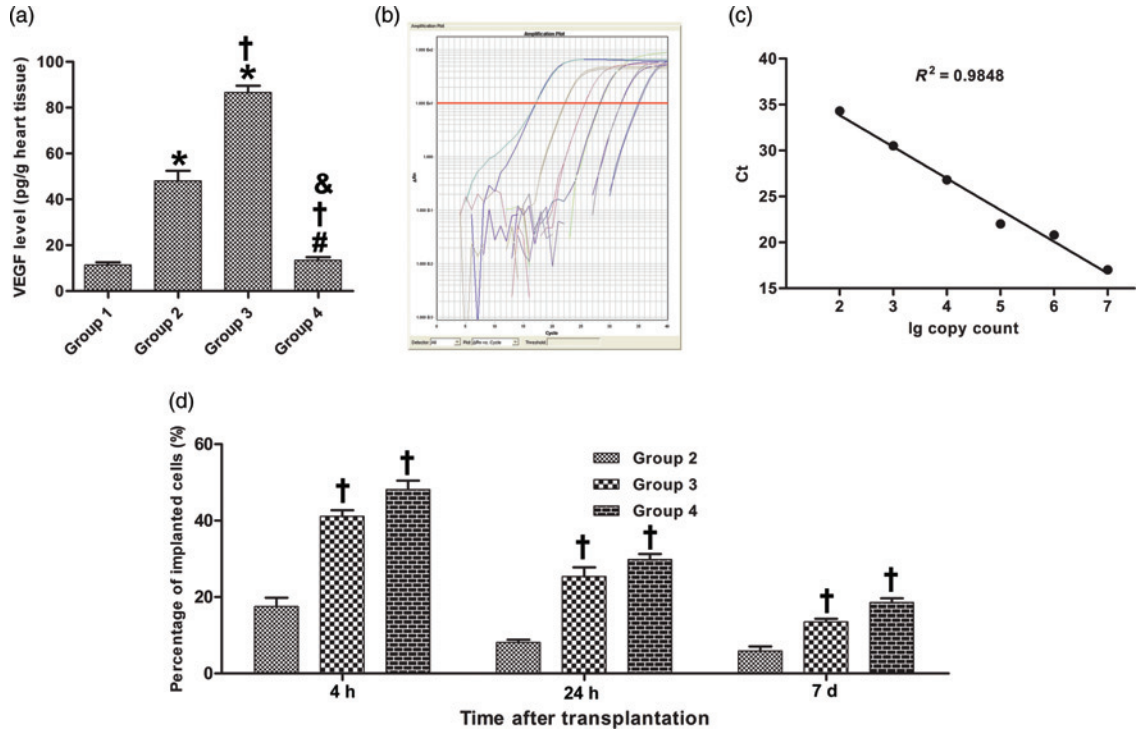

Figure 4 Myocardial VEGF concentration and graft MSC survival. (a) Myocardial VEGF concentration after infarction. (b) Realtime amplification plot of Sry gene standard. (c) Standard curve generated from data in (b) showing relationship between threshold cycle (Ct) value and number of Sry gene copies. (d) Percentage of graft MSCs in Groups 2, 3 and 4 at four hours, 24 h, and seven days after transplantation. (*Versus Group 1, $P < 0.01$; $^\#$versus Group 1, $P > 0.05$; †versus Group 2, $P < 0.01$; $^\&$versus Group 3, $P < 0.01$). VEGF, vascular endothelial growth, MSC, mesenchymal stem cell. (A color version of this figure is available in the online journal)

Heart function and angiogenesis

At four weeks after infarction, LVEF and LVFS in Group 1 were $36.81 \pm 1.55\%$ and $17.56 \pm 0.86\%$, respectively. A significant improvement in LVEF and LVFS was observed in Group 2 ($49.09 \pm 0.78\%$ and $24.41 \pm 0.52\%$) and Group 3 ($57.46 \pm 2.56\%$ and $29.73 \pm 1.68\%$). Furthermore, LVEF and LVFS in Group 3 showed greater improvement than those in Group 2 ($P < 0.05$). Both LVIDed and LVIDes increased for all groups. LVIDed and LVIDes in Group 3 (3.69 ± 0.13 mm, 2.69 ± 0.13 mm) were shorter than those in Group 2 (4.03 ± 0.09 mm, 3.05 ± 0.09 mm, $P < 0.05$) and Group 1 (4.46 ± 0.12 mm, 3.68 ± 0.12 mm, $P < 0.01$). LVAWTed and LVAWTes were best maintained in Group 3 (0.76 ± 0.02 mm, 1.17 ± 0.03 mm), followed by Groups 2 and 1, which showed thinner LVAWTed (0.70 ± 0.02 mm, $P < 0.05$; 0.49 ± 0.01 mm, $P < 0.01$) and LVAWTes (0.94 ± 0.03 mm, 0.59 ± 0.02 mm, $P < 0.01$). LVEF and LVFS in Group 2 ($44.43 \pm 2.55\%$, $21.79 \pm 1.46\%$) and in Group 3 ($54.09 \pm 2.94\%$, $27.64 \pm 1.86\%$) improved significantly compared with those in Group 1 ($33.79 \pm 2.30\%$, $16.00 \pm 1.21\%$, $P < 0.01$). Furthermore, LVEF and LVFS in Group 3 showed greater enhancement than those in Group 2 ($P < 0.05$). In addition, Group 4 demonstrated the following parameters (LVEF: $80.48 \pm 1.34\%$, LVFS: $49.20 \pm 1.69\%$, LVIDed: 2.76 ± 0.05 mm, LVIDes: 1.87 ± 0.05 mm, LVAWTed: 0.82 ± 0.01 mm, LVAWTes: 1.30 ± 0.04 mm) (Figure 5a–f).

As shown in representative micrographs of sections from each treatment group (Figure 5g), an increased number of capillaries based on PECAM-1 immunostaining were present in Group 3 than in Groups 1 or 2. The mean capillaries positive for PECAM-1 per high-power fields in Group 3 (93.50 ± 2.84) were significantly higher than those in Group 1 (55.38 ± 2.64), Group 2 (30.13 ± 3.17) and Group 4 (40.25 ± 2.63) ($P < 0.01$) (Figure 5h).

Discussion

As a critical part of clinical gene therapy for cardiac repair, the ideal VEGF gene delivery system should be effective in transfection efficiency, biocompatible in cytotoxicity and controllable in gene expression. In this study, we constructed a novel gene delivery system which comprised hypoxia-regulated VEGF plasmids and hyperbranched dendrimer-based gene vehicle. The hPAMAM-pHRE-$hVEGF_{165}$ system-manipulated MSCs were then transplanted into mice ischemic myocardium and promoted cardiac repair successfully.

It was reported that MSCs from different strains differed in hematopoietic stem cell marker CD34. Although human MSCs were negative for CD34, C57/Bl6 MSCs expressed high levels of CD34, FVB/N MSCs expressed moderate levels, and both BALB/c and DBA1 MSCs expressed low levels.[23] In our study, we isolated MSCs from C57/BL6 mice. Flow cytometry results demonstrated that MSCs were positive (91.67%) for CD34. Therefore, the purity of MSCs in this study was high, which could be applied for studies properly.

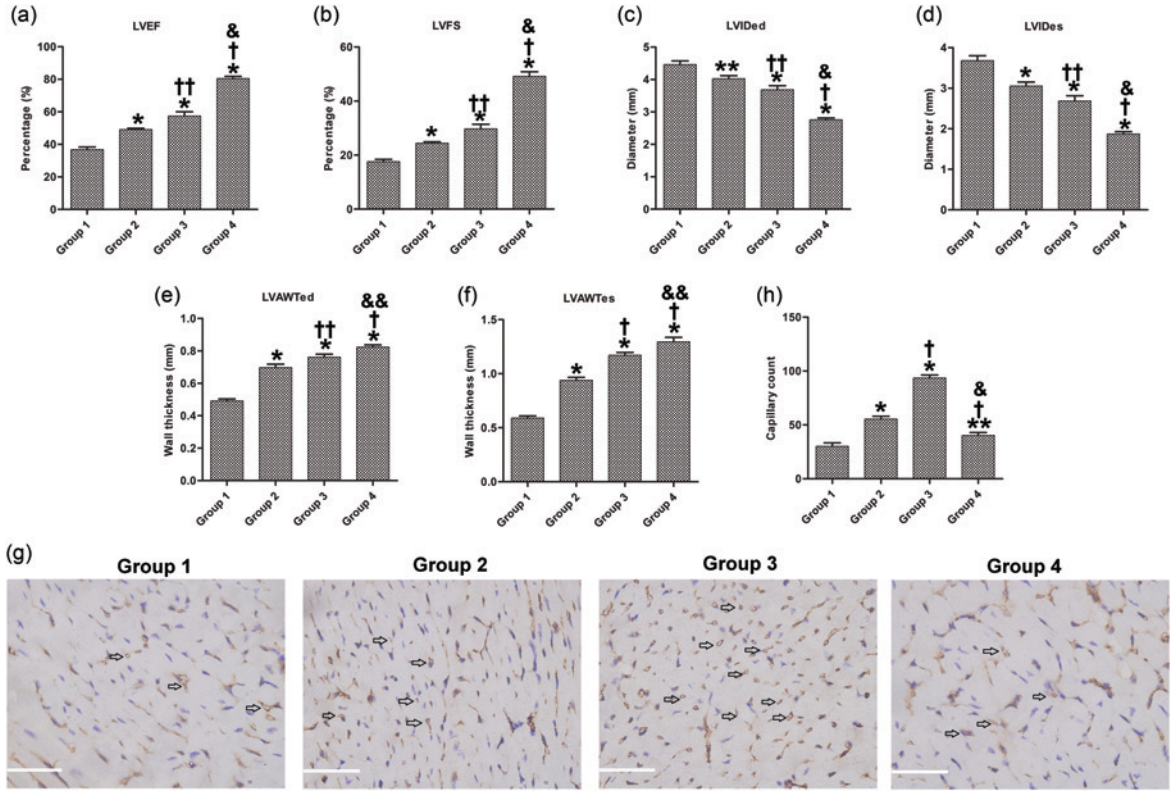

Figure 5 Heart function and angiogenesis assessment. (a–f) LVEF, LVFS, LVIDed, LVIDes, LVAWTed and LVATWTes of Groups 1, 2 and 3. (g) Capillaries at the peri-infarct area based on PECAM-1 immunostaining (arrow). (h) The comparison of capillary count of Groups 1, 2 and 3. (*Versus Group 1, $P < 0.01$; **versus Group 1, $P < 0.05$; †versus Group 2, $P < 0.01$; ††versus Group 2, $P < 0.05$; $^{\&}$versus Group 3, $P < 0.01$; $^{\&\&}$versus Group 3, $P < 0.05$). Scale bar = 50 μm (g). LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; LVIDed, left ventricular internal diameter at end-diastole; LVIDes, left ventricular internal diameter at end-systole; LVAWTed, left ventricular anterior wall thickness at end-diastole; LVATWTes, left ventricular anterior wall thickness at end-systole. (A color version of this figure is available in the online journal)

A potential problem associated with prolonged and high-level expression of VEGF is the formation of hemangioma.[6] Although we did not see such a complication in our previous experiments on mice, for clinical application, it is important to safeguard against this complication. In this study, we applied hypoxia response element to control $hVEGF_{165}$ expression *in vitro* and *in vivo*. We chose to use five copies of hypoxia response element in the study because previous studies demonstrated that five copies of hypoxia response element yielded the best induction of gene expression under hypoxia condition.[24,25] Although higher VEGF gene expression (increased by 16.4-folds) has been reported by Su *et al.*,[11] who inserted nine copies of hypoxia response element into VEGF plasmids, this could be due to high gene transfection efficiency (>90%) related to adenoviral-associated virus. Therefore, pHRE-$hVEGF_{165}$ will provide a safer alternative to the conventionally available VEGF plasmids. In addition, plasmids with similar sizes contribute equally to gene transfection efficiency.[26] In our study, the size of pHRE-$hVEGF_{165}$ (4.25 kb) was similar to that of pEGFP (4.7 kb), which enable pEGFP to monitor transfection efficiency of hPAMAM *in vitro*.

An ideal gene vector system should: (i) demonstrate high gene-loading capability and ease of manufacturing; and (ii) ensure a high level of gene transfection and expression with biocompatibility and immunogenicity.[27] Using the optimized one-pot method, the synthesis process of hPAMAM is much simpler and faster, and the raw materials of hPAMAM, MA and DETA are easy to access for manufacture. These advantages could contribute to the widespread application of hPAMAM. Moreover, as an analog to PAMAM, hPAMAM demonstrated a hyperbranched and multivalent functionalized terminal surface.[20,21] We speculated that the amino terminals at the periphery of hPAMAM could also be protonated at physiological pH, form complexes with the anionic deoxyribonucleic acid of DNA molecules and promote cellular uptake by condensing DNA into nanosized particles, which is similar to the endocytosis process of other polymer vehicles. After endocytosis, the amino groups may act as a proton sponge in endosomes, thus enhancing the release of DNA into the cytoplasm.[28] Compared with PEI ($22.69 \pm 1.73\%$) and Lipofectamine 2000 ($31.44 \pm 0.85\%$), hPAMAM has better transfection efficiency ($38.98 \pm 1.95\%$) in primary MSCs. hPAMAM also showed excellent cell viability ($92.38 \pm 1.09\%$) during transfection under optimal transfection condition. Hence, hPAMAM is an economical, effective and biocompatible gene delivery vehicle.

Using hPAMAM as a gene vehicle to deliver pHRE-$hVEGF_{165}$ into MSCs, our study demonstrated the stable expression of $hVEGF_{165}$ protein under hypoxia *in vitro*. It was reported that VEGF over-expression for 1–2 weeks might be sufficient to form collateral vessels in ischemic myocardium.[29] In the present study, the

transfected MSCs could express $hVEGF_{165}$ stably for two weeks, with the peak expression at day 2. The time-course experiments showed that HUVEC proliferation was statistically enhanced by $hVEGF_{165}$ secreted from the transfected MSCs, which demonstrated that this hPAMAM-pHRE-$hVEGF_{165}$ gene delivery system could promote endothelial propagation *in vitro*.

The transplantation of hPAMAM-pHRE-$hVEGF_{165}$ gene delivery system-manipulated MSCs could enhance ischemic myocardium VEGF concentration, which improved the graft MSC survival, increased neovascularization and ultimately preserved cardiac function to a significantly greater degree than non-transfected MSC transplantation. In this study, the rationale for choosing the acute phase of myocardial infarction as the time for MSC transplantation is based on the following facts: (i) the paracrine action from graft stem cells can salvage ischemic myocardium at the early phase, and (ii) clinical application of stem cell therapy currently exists in most large heart hospitals as a supplemental treatment to angioplasty patients with acute myocardial infarction.[30] Therefore, protecting graft cells from acute death in ischemic myocardium is important for clinical applications. Acute myocardium infarction will cause severe ischemia, followed by an inflammation response, which significantly reduces the graft cell survival rate.[31] 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. Realtime PCR 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 fields from representative tissue sections.[32] Our study demonstrated that graft cell survival improved significantly after implantation of hPAMAM-pHRE-$hVEGF_{165}$-transfected MSCs, which may result from a direct effect of enhanced VEGF concentration on the transplanted cells via VEGF receptor-2 as for endothelial cells and improved blood supply to grafted myoblasts through both vasodilation and enhanced angiogenesis.[33] Furthermore, immunostaining for capillaries in the ischemic zone revealed that neovascularization was more pronounced around the injection sites in the infarct border zone at 28 days after infarction. Although the decreased risk for hemangioma formation could not be observed directly in this study, the hPAMAM-pHRE-$hVEGF_{165}$ gene delivery system provides us with a safer strategy for clinical VEGF gene therapy.

Conclusions

In summary, we developed hPAMAM-pHRE-$hVEGF_{165}$ as an economical gene delivery system with high transfection efficiency, low cytotoxicity and controllable gene expression. After myocardium infarction, treatment with hPAMAM-$hVEGF_{165}$-transfected MSCs improved myocardial VEGF concentration, which improved graft MSC survival, increased neovascularization and ultimately improved heart function. This novel VEGF gene delivery system is feasible and may have clinical relevance for tissue repair in ischemic diseases.

Author contributions: CG, CW and KZ designed the study; KZ, HL and CG conducted the experiments; KZ, HL and DX analyzed the data; and KZ and HL wrote the manuscript. KZ and HL contributed equally to this work.

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