Transplantation of novel vascular endothelial growth factor gene delivery system manipulated skeletal myoblasts promote myocardial repair

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A B S T R A C T
Background: Skeletal myoblast (SkM) transplantation combined with vascular endothelial growth factor (VEGF) gene delivery has been proposed as a promising therapy for cardiac repair. Nevertheless, the defective gene vectors and unregulable VEGF expression in vivo hinder its application. Therefore, the search for an economical, effective, controllable gene delivery system is quite necessary.

Methods: In our study, hyperbranched polyamidoamine (h-PAMAM) dendrimer was synthesized as a novel gene delivery vector using a modified method. And hypoxia-regulated human VEGF-165 plasmids (pHRE-hVEGF165) were constructed for controllable VEGF gene expression. The efficiency and feasibility of h-PAMAM-HRE-hVEGF165 gene delivery system manipulated SkM transplantation for cardiac repair were investigated in myocardial infarction models.

Results: The h-PAMAM encapsulated pHRE-hVEGF165 could resist nuclease digestion for over 120 min. In primary SkMs, h-PAMAM-pHRE-hVEGF165 gene delivery system showed high transfection efficiency (43.47 ± 2.22%) and minor cytotoxicity (cell viability = 91.38 ± 0.48%). And the transfected SkMs could express hVEGF165 for 18 days under hypoxia in vitro. For myocardial infarction models, intramyocardial transplantation of the transfected SkMs could result in reduction of apoptotic myocardiocytes, improvement of grafted cell survival, decrease of infarct size and interstitial fibrosis, and increase of blood vessel density, which inhibited left ventricle remodeling and improved heart function at the late phase following infarction.

Conclusions: These results indicate that h-PAMAM based pHRE-hVEGF165 gene delivery into SkMs is feasible and effective, and may serve as a novel and promising gene therapy strategy in ischemic heart disease.

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1. Introduction

Vascular endothelial growth factor (VEGF) gene delivery combined with skeletal myoblast (SkM) transplantation has been proposed as a promising therapy strategy for cardiac repair after myocardium infarction [1]. The VEGF manipulated SkMs could survive in the ischemic myocardium for weeks and promote cardiac repair eventually [2,3].

However, the VEGF gene delivery system, including gene vector and VEGF plasmid, still needs to be optimized. On the one hand, development of an economical, effective and biocompatible gene vector is necessary for clinical application. Viral gene vectors can deliver therapeutic gene into cells with high transfection efficiency [4]. However, immunogenicity and oncogenic potential of this method severely hinder its clinical application [5]. Non-viral vector gene delivery approach provides a safer alternative to overcome these untoward effects of viral vectors. It’s regrettable that most non-viral vectors demonstrate low gene transfection efficiency and high cytotoxicity [6]. Among the numerous non-viral vectors investigated, polyamidoamine (PAMAM) dendrimer has attracted a lot of attention because of its minor cytotoxicity and encouraging transfection efficiency [7,8]. However, PAMAM synthesis procedure requires stepwise convergent approaches and repeated purification steps, which is extremely time-consuming and costly [9]. Wu et al. synthesized hyperbranched PAMAM (h-PAMAM) dendrimer by polycondensation...
of N-[2-((1-piperazinyl)ethyl]-1, 2-ethanediamine and maleic anhydride. They reported that the resulting h-PAMAM was structurally analogous to PAMAM and showed excellent DNA protection ability, low cytotoxicity and high gene transfection efficiency in cell lines [10]. Gao and Yan developed a simple synthesis technique called “one-pot method”, which could achieve h-PAMAM by polycondensation of methyl acrylate and diethylentriamine [11]. In an earlier study, we modified the “one-pot method” to improve the reliability of synthesis procedure, and investigated detailed chemical characteristics of h-PAMAM nanoparticles [12]. Up to now, no studies have reported h-PAMAM as a gene vector in primary stem cells. Therefore, h-PAMAM based gene delivery was investigated in our present study.

On the other hand, VEGF gene therapy, combined with SkM transplantation, successfully initiates angiogenic response to ischemic myocardium [13]. Given that ischemic heart disease is recurring and progressive, and long-term uncontrolled VEGF overexpression may result in angiofibroma formation, more accurately controlled VEGF expression is expected to improve prognosis and abrogate undesired effects [14]. Hypoxia inducible factor-1α (HIF-1α) is a master regulator of multiple angiogenic growth factors that are expressed in response to tissue hypoxia. The hypoxia response element is a DNA fragment which could bind HIF-1α at the transcriptional level and launch the downstream-gene expression [15–17]. We hypothesized that this element could be inserted into the promoter region of human VEGF165 (hVEGF165) gene to form hypoxia-regulated hVEGF165 (HRE-hVEGF165) gene for controllable VEGF expression. Under hypoxia condition, HIF-1α could bind to the element and up-regulate VEGF gene expression. And when the regional blood supply is adequate, VEGF expression could be turned off by the normoxia condition [18]. Therefore, HRE-hVEGF165 is a good candidate for controlling VEGF expression in ischemic myocardium.

In the present study, we optimized the delivery of h-PAMAM based HRE-hVEGF165 gene into primary SkMs, and grafted the genetically-manipulated SkMs into infarct myocardium. We anticipate that this novel gene delivery system combined with SkM mediated cellular therapy will enable a new approach for cardiac repair.

2. Materials and methods

2.1. Preparation of h-PAMAM–DNA complexes

The synthesis of h-PAMAM was carried out by our modified “one-pot method” [12]. Full details on the synthesis procedure can be found in the Supplementary material. The hVEGF165 gene was cloned into pGEM-easy plasmid (Promega, Madison, WI, USA). Then cDNA fragment containing hVEGF165 was subcloned into pGL-3-Promoter vector (Promega) to replace luciferase gene. Five copies of hypoxia response element (Gene Chem, Shanghai, China) were inserted into the pGL-3 plasmid upstream of the SV40 promoter. Thus, the hypoxia-regulated plasmid containing hVEGF165 under the control of HRE and SV40 promoter (pHRE-hVEGF165) was derived. Enhanced green fluorescent protein plasmid (pEGFP) was purchased from Gene Chem. The h-PAMAM and 2 μg DNA (pEGFP or pHRE-hVEGF165) were dialyzed separately in 50 μl NaCl (150 mM). The diluted DNA was then added to the nanoparticle solution at different weight ratios (wh-PAMAM/wDNA = 1 to 15) and immediately vortexed gently, then allowed to sediment for 10 min. The complexes were used immediately for experiments.

2.2. Characteristics of the h-PAMAM–DNA complexes

The particle size distribution and zeta potential of h-PAMAM–DNA complexes at different weight ratios were detected using a Zetasizer (Malvern, Worcestershire, UK). The shape of the complexes was visualized using transmission electron microscopy (TEM, JEM, JEM-1230, Tokyo, Japan). For determining the zeta potential of transfected cells under repeated hypoxia and hypoxia condition, the efficiency of this transgene system in response to recurring ischemia, the expression profile of transfected cells under repeated hypoxia and hypoxia cycles was evaluated by real-time PCR. At regular 2 day intervals (from day 0 until day 18) after transfection, the culture supernatant sample of SkMs-hVEGF under hypoxia was collected for hVEGF165 protein quantification using a hVEGF165 enzyme-linked immunosorbent assay (ELISA) kit (R&D) according to the supplier’s instructions. The hVEGF165 protein secreted by SkMs-hVEGF under normoxia, and SkMs-0 under normoxia and hypoxia were used as controls.

2.5. hVEGF165 Expression in vitro

The h-PAMAM-pHRE-hVEGF165 transfected SkMs (SkMs-VeGF) were seeded onto chamber slides and incubated under normoxia or hypoxia condition. The nontransfected SkMs (SkMs-0) were used as a control. The next day, samples were immunostained using anti-human VEGF primary antibody (R&D, Santa Fe Springs, CA, USA), and 4, 6-diamidino-2-phenylindole (DAPI) was used for nuclear counterstaining [1]. The immunostained cells were visualized using a confocal microscope (Olympus, Tokyo, Japan).

After transfection, SkMs-VeGF were cultured for 18 days and then harvested to detect the intracellular residual complexes under TEM. SkMs-VeGF cells under hypoxia for 2, 4, 8 and 18 days after transfection were collected to quantify hVEGF165 gene expression using real-time polymerase chain reaction (PCR) [1]. To determine the efficiency of this transgene system in response to recurring ischemia, the expression profile of transfected cells under repeated hypoxia and hypoxia cycles was evaluated by real-time PCR.

2.6. Myocardial infarction model and cell transplantation

Before cell transplantation, SkMs were labeled with DAPI culture medium (2 μg/ml) for 24 h at 37 °C in a 5% CO2 incubator. Adult, female C57/Bl6 mice (6–8 weeks old) weighing 20 to 25 g were anesthetized by intraperitoneal administration with a mixture of ketamine hydrochloride (40 mg/kg) and diazepam (5 mg/kg). Mice were endotracheally intubated and mechanically ventilated on a small animal ventilator (KDS model 35, KDScientific, Holliston, MA, USA) with supplemental oxygen. The myocardial infarction models were created by permanently ligating the left anterior descending coronary artery with 8–0 polypropylene suture, 2 mm distal to the left auricle. Ten minutes later, 50 μl DMEM without SkMs (Group 1), or with 1 × 10^6 SkMs-0 (Group-2), or with SkMs-VeGF (Group-3) were intramyocardially injected into three sites of the peri-infarct region.

2.7. In situ cardiomyocytes apoptosis

One day after cell transplantation, hearts (n = 3 in each group) were harvested to detect apoptotic cardiomyocytes using the TUNEL detection kit (Roche, Basel, CH, USA). For the apoptotic index, tissue sections were counterstained with propidium

iodide (PI) after TUNEL. Total number of cell nuclei and apoptotic nuclei were counted in four fields of the peri-infarct area per slide, and the apoptotic index was calculated as the percentage of TUNEL positive apoptotic nuclei to total nuclei per field.

2.8. Differentiation and survival of gifted cells

At 28 days after cell transplantation, surviving SkMs in vivo were observed directly under a fluorescence microscope. Skeletal myotube formed by surviving SkMs in Group 2 and 3 (n = 3 in each group) were immunostained with a 1:50 dilution of anti-skeletal myosin heavy chain antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). This was followed by incubation with a 1:100 dilution of Cy-3 labeled secondary antibody.

The amount of surviving male donor SkMs was evaluated using real-time PCR by amplifying the sex-determining region of the Y-chromosome (Sry) gene [20]. Briefly, Sry gene was extracted from male skeletal myoblasts and cloned in Escherichia coli. After extraction of plasmids, gene sequencing was performed to confirm the Sry gene. And then the Sry plasmids were used as a quantification standard after serial dilution. Real-time PCR analysis was performed using the SYBR Green kit (QIAGEN, Dusseldorf, GER). The primers were as follows: (forward) 5′-CGTGGCCAAGCCGAAGATG-3′; (reverse) 5′-CCGTT CATTGGCCCTGAATC-3′. The Sry gene copy number in Group 2 and Group 3 was determined at 10 min, day 1, day 3, day 7 and day 28 (n = 5 for each time-point in each group) after gender-mismatch transplantation.

2.9. Heart function

At 4 weeks after infarction, heart function (n = 10 animals each group) was measured using an echocardiograph (Veo770, VisualSonics, Toronto, Canada) by an investigator blinded to the therapeutic intervention on animals. From M-mode echocardiogram, measurements were obtained for left ventricular anterior wall thickness at end-diastole (LVAWTed), end-systole (LVAWTes), left ventricular inter-echocardiogram, measurements were obtained for left ventricular anterior wall thickness at end-diastole (LVAWTed), end-systole (LVAWTes), left ventricular inter-

2.10. Infarct size and interstitial fibrosis

After heart function assessment, hearts (n = 5 in each group) were sectioned into 5 segments parallel to the apex-base axis and stained with Masson trichrome. All sections were projected onto a screen for computer-assisted planimetry. The scar length to left ventricular circumference ratio of the endocardium and epicardium was expressed as a percentage to determine infarct size. The interstitial fibrosis in non-infarct area was evaluated as collagen volume fraction. All data were calculated using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA).

2.11. Angiogenesis

Hearts (n = 5 in each group) were harvested to evaluate angiogenesis after echocardiography. The myocardial arteriolar and capillary densities were measured by staining for alpha-smooth muscle actin (α-SMA) and PECAM-1 (Santa Cruz Biotechnology) (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.

2.12. Statistical analysis

The data were analyzed with SPSS 17.0 software (SPSS, Chicago, IL, USA). All values were expressed as the mean ± 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 the Student's t-test.

3. Results

3.1. Synthesis of h-PAMAM and characterization of h-PAMAM–DNA complexes

GPC (Fig. 1A) showed that the weight-average molecular weight was 6.55 × 10^6, the number-average molecular weight was 3.80 × 10^6, and the molecular weight polydispersity index was 1.72. The molecular structure of h-PAMAM was confirmed using FTIR (Fig. 1B).

When combined with pEGFP, the average particle size and zeta potential for various h-PAMAM/wDNA ratios were shown in Fig. 1C. TEM images showed the h-PAMAM–DNA appeared either oval- or spindle-shaped between 100 and 500 nm, with a h-PAMAM/wDNA ratio of 8 using 2 μg DNA (Fig. 1D). The encapsulated DNA could be protected from degradation by h-PAMAM for over 120 min, in contrast to the naked DNA, which was fully degraded by DNase I during 30 min (Fig. 1E).

3.2. Optimization of h-PAMAM–DNA complex transfection into SkMs

Desmin was expressed specifically in SkMs (Fig. 2A, B), and flow cytometry showed that cell purity was 92.59 ± 1.29% (Fig. 2C).

Maximum transfection efficiency (43.47 ± 2.22%) could be achieved when h-PAMAM/wDNA ratio was 8 using 2 μg DNA per 1 × 10^6 cells and was associated with low cytotoxicity (cell viability = 91.38 ± 0.48%) (Fig. 2D). Alteration of DNA quantity did not increase transfection efficiency at h-PAMAM/wDNA ratio of 8 (Fig. 2E). Therefore, the optimal transfection condition was h-PAMAM/wDNA of 8 using 2 μg DNA per 1 × 10^6 cells. Under this condition, h-PAMAM could achieve higher transfection efficiency than Lipofectamine 2000 (31.9 ± 1.53%, p < 0.05) and PEI (13.43 ± 0.77%, p < 0.01). The cell viability after h-PAMAM based transfection was similar to PEI (90.30 ± 0.47%, p = 0.185) but higher than Lipofectamine 2000 (72.34 ± 1.26%, p < 0.01) (Fig. 2F–H).

3.3. Gene expression under optimal transfection condition

Immunostaining showed that SkMs-VEGF could express hVEGF165 under hypoxia condition (Fig. 3A–C). Under TEM, the intracellular h-PAMAM–hPRE–hVEGF165 complexes were observable at 18 days after transfection (Fig. 3D). Under hypoxia, gene expression of SkMs-VEGF increased 3.41 ± 0.3 times at day 2, 3.39 ± 0.47 times at day 4, 1.94 ± 0.17 times at day 8 and 1.53 ± 0.36 times at day 18 compared with that of SkMs-0 (Fig. 3E). Gene expression was up-regulated by hypoxia and down-regulated by normoxia (Fig. 3F). ELISA demonstrated that SkMs-VeGF secreted hVEGF165 stably for 18 days, with the peak expression (2528.59 ± 67.09 pg/ml) at day 2 after transfection (Fig. 3G).

3.4. Apoptosis

The apoptotic cardiomyocytes in the peri-infarct area were stained using TUNEL (Fig. 4A–I). The percentage of TUNEL-positive cells was lowest (29.68 ± 1.62%) in Group 3 (Group 1: 53.96 ± 1.61%; Group 2: 42.83 ± 1.67%) (p < 0.01) (Fig. 4).

3.5. Survival of grafted SkMs

SkMs were labeled with DAPI successfully in vitro (Fig. 5A, B). In vivo, the surviving grafted SkMs could differentiate into multinucleated skeletal myotubes which were stained positively for skeletal myosin heavy chain (Fig. 5C–F). The survival rate of grafted SkMs in Group 3 was significantly higher than that in Group 2 (10 min: 41.12 ± 1.58% vs. 32.23 ± 1.93%, p < 0.05; day 1: 15.53 ± 1.52 vs. 9.02 ± 1.38%, p < 0.05; day 2: 7.03 ± 2.14% vs. 11.16 ± 1.27%, p < 0.05; day 7: 32.32 ± 2.51% vs. 13.67 ± 1.78%, p < 0.01; day 28: 38.63 ± 2.98 vs. 23.58 ± 1.37%, p < 0.05) (Fig. 5G–I).

3.6. Heart function

Echocardiography showed that both LVIded and LVIdes increased for all groups. LVIded and LVIdes in Group 3 (3.75 ± 0.09 mm, 2.72 ± 0.12 mm) were shorter than those in Group 2 (4.00 ± 0.09 mm, 3.10 ± 0.10 mm, p < 0.05) and 1 (4.33 ± 0.11 mm, 3.65 ± 0.12 mm, p < 0.01). LVAWted and LVAWtes were best maintained in Group 3 (0.78 ± 0.05 mm, 1.14 ± 0.03 mm), followed by Group 2 and 1, which showed thinner LVAWted (0.71 ± 0.02 mm, 0.48 ± 0.02 mm, p < 0.01) and LVAWtes (0.96 ± 0.03 mm, 0.60 ± 0.02 mm, p < 0.01).
Fig. 1. Characterization of h-PAMAM–DNA complexes. A, molecular weight of tertiary amine terminated h-PAMAM measured by GPC. B, FTIR spectra of h-PAMAM. C, average particle size and zeta potential of h-PAMAM–DNA at various \( \text{w}_{\text{h-PAMAM}}/\text{w}_{\text{DNA}} \) ratios using 2 \( \mu \text{g DNA} \). D, TEM image of h-PAMAM–DNA nanoparticles at \( \text{w}_{\text{h-PAMAM}}/\text{w}_{\text{DNA}} \) ratio of 8 using 2 \( \mu \text{g DNA} \). E, h-PAMAM encapsulated DNA showed stability against DNase I for up to 120 min as compared with the naked DNA. Scale bar = 500 nm.

Fig. 2. Optimization of h-PAMAM based gene transfection. A, SkMs demonstrated desmin expression (red) by immunofluorescence. Nuclei were counterstained blue with Hoechst 33342 (blue). B, fibroblasts were used as a negative control. C, 92.59 ± 1.29% of isolated cells was positive for desmin expression using non-stained cells as a control for auto-fluorescence. D, gene transfection efficiency and viability of SkMs when \( \text{w}_{\text{h-PAMAM}}/\text{w}_{\text{DNA}} \) ratios varied from 1 to 15 using 2 \( \mu \text{g pEGFP per 10}^5 \) cells. E, gene transfection efficiency and cell viability when pEGFP quantity varied from 2 \( \mu \text{g to 5} \mu \text{g at w}_{\text{h-PAMAM}}/\text{w}_{\text{DNA}} \) ratio of 8. Flow cytometry demonstrated the comparison of transfection efficiency (F) and cytotoxicity (G) of h-PAMAM, Lipofectamine 2000 and PEI. H, compared with Lipofectamine 2000 and PEI, h-PAMAM could achieve higher transfection efficiency under optimal transfection conditions with minor cytotoxicity (\( °\text{vs. Lipofectamine 2000, p < 0.01; °°vs. Lipofectamine 2000, p < 0.05; °°vs. PEI, p < 0.01; °vs. PEI, p = 0.185} \)). Scale bar = 50 \( \mu \text{m} \) (A and B).
LVEF and LVFS in Group 2 (44.43 ± 2.55%, 21.79 ± 1.46%) and in Group 3 (54.09 ± 2.94%, 27.64 ± 1.86%) improved significantly compared with those in Group 1 (33.79 ± 2.30%, 16.00 ± 1.21%, \( p < 0.01 \)). Furthermore, LVEF and LVFS in Group 3 showed greater enhancement than those in Group 2 (\( p < 0.05 \)). LVAWTP in Group 3 was higher (47.92 ± 4.93%) than that in Group 2 (35.68 ± 1.57%, \( p < 0.05 \)) and 1 (24.96 ± 1.76%, \( p < 0.01 \)). LVedV in Group 3 (60.42 ± 3.52 \( \mu l \)) was smaller than that in Group 1 (83.88 ± 5.57 \( \mu l \)) and 2 (69.33 ± 4.76 \( \mu l \)), though no significant difference was observed between Group 1 and 2 (\( p = 0.067 \)), or between Group 2 and 3 (\( p = 0.155 \)). LVesV in Group 1 (60.25 ± 5.00 \( \mu l \)) and 2 (38.53 ± 3.00 \( \mu l \)) increased significantly compared with Group 3 (28.17 ± 2.87 \( \mu l \), \( p < 0.05 \)) (Fig. 6A-1).

### 3.7. Infarct size and interstitial fibrosis

At 28 days after infarction, Masson trichrome of myocardium from three groups was shown in Fig. 7A-I. Infarct size and interstitial fibrosis of non-infarct area were significantly reduced in Group 3 (23.57 ± 1.73%, 5.02 ± 0.16%) compared with Group 1 (50.00 ± 2.41%, 11.42 ± 0.7%) and 2 (35.02 ± 1.55%, 7.39 ± 0.36%) (\( p < 0.01 \)) (Fig. 7J).

### 3.8. Angiogenesis

The arteriole count based on \( \alpha \)-SMA and capillary count based on PECAM-1 immunostaining in Group 3 (4.83 ± 0.31 per field, 82.17 ± 3.37 per field) was higher than those in Group 1 (0.67 ± 0.33 per field) and 2 (3.78 ± 0.27 per field, 74.38 ± 3.73 per field) (Fig. 7K).
field, 30.00 ± 2.58 per field) and 2 (2.00 ± 0.26 per field, 51.17 ± 2.83 per field) at 4 weeks after infarction (p < 0.01) (Fig. 8A–J).

4. Discussion

The effective, biocompatible and controllable gene therapy is quite necessary for clinical application. In our study, h-PAMAM nanoparticle based pHRE-hVEGF165 gene delivery system was developed and applied in SkM based transfection. And the manipulated SkM transplantation could promote cardiac repair after infarction.

Compared with the complicated synthesis process of PAMAM, this modified “one-pot” synthesis method of h-PAMAM is economical and reliable, which may be good for large-scale production. As a cationic polymer, h-PAMAM could be a promising gene vector with excellent protection for plasmids, high transfection efficiency and low cytotoxicity. In our study, DNA could be encapsulated by h-PAMAM and protected from DNase I digestion for up to 120 min. However, intracellular nuclease concentration is markedly lower than that used in this study. Hence, it is concluded that h-PAMAM–DNA could resist nuclease digestion after transfection. It is known that high transfection efficiency contributes to high gene expression, and is critical for an effective gene delivery system [21]. In our present study using primary SkMs, h-PAMAM also showed higher transfection efficiency (43.47 ± 2.22%) than Lipofectamine 2000 and PEI (25 kDa), which have been applied as commercial non-viral vectors. It is well known that low transfection efficiency is one of the big obstacles to non-viral vector-mediated gene delivery into primary SkMs. And the gene transfection expression efficiencies of non-viral vector-mediated gene transfer may be influenced by particle size and zeta potential [22]. Previous studies demonstrated that size-dependent endocytosis included clathrin- and caveolae-mediated endocytosis pathways. And nanoparticle with a diameter of <200 nm was taken up predominantly via clathrin-mediated endocytosis. In our study, at the WH/PAMAM/WDNA ratio of 8 using 2 μg DNA, the particle size was 150.76 ± 0.94 nm. This particle size may promote optimal endocytosis through the clathrin-mediated pathway, which also brought maximum transfection efficiency. However, detailed mechanism of the suitable particle size (150.76 ± 0.94 nm) for maximum endocytosis needs further investigation in future study. In addition, it was found that higher zeta potential (<26 mV) could bind more h-PAMAM–DNA complexes to cell membrane and result in higher transfection efficiency. But zeta potential above 26 mV could not give more
Fig. 5. Graft survival. A, DAPI labeled SkMs in vitro. B, the light microscopy of (A), surviving SkMs in myocardium of Group 2 (C) and 3 (D). Immunostaining of skeletal myosin heavy chain (red) revealed myogenic differentiation of DAPI labeled SkMs (blue) in Group 2 (E) and 3 (F). G, real-time amplification plot of Sry gene standard. H, standard curve generated from data in (C) showing the relationship between threshold cycle (Ct) and number of male SkMs. I, Survival rate of grafted SkMs in Group 2 and Group 3 at 10 min, day 1, day 3, day 7 and day 28 after transplantation (* vs. Group 2, p < 0.01; ** vs Group 2, p < 0.05). Scar bar = 100 μm (A and B), 200 μm (C and D) and 50 μm (E and F).

Fig. 6. Heart function (A–I). Heart function assessment was evaluated on three groups (* vs. Group 1, p < 0.01; ** vs. Group 1, p < 0.05; † vs. Group 2, p < 0.01; †† vs. Group 2, p < 0.05; ‡ vs. Group 1, p = 0.067; # vs. Group 2, p = 0.153).
binding rate and higher transfection efficiency [1,23]. In our study, zeta potential of complexes achieved 32.58 ± 0.96 mV at the \(W_{\text{h-PAMAM}}/W_{\text{DNA}}\) ratio of 8, and higher zeta potential (>32.58 ± 0.96 mV) as well as higher \(W_{\text{h-PAMAM}}/W_{\text{DNA}}\) ratio (>8) would not enhance the transfection efficiency significantly, since zeta potential of 26 mV is sufficient for binding the h-PAMAM–DNA complexes to cell membrane for endocytosis. Moreover, it is essential to consider the biocompatibility of gene vectors for clinic application [24]. Under the optimal transfection condition, h-PAMAM showed excellent biocompatibility (cell viability = 94.6 ± 1.4%) as PEI (25 kDa) (cell viability = 90.30 ± 0.47%), while Lipofectamine 2000 showed high cytotoxicity during transfection (cell viability = 72.34 ± 1.26%).

Using h-PAMAM as gene vector to deliver pHRE-hVEGF165 plasmids into SkMs, our study demonstrated flexible and stable hVEGF165 expression in vitro. Controllable VEGF expression is significantly important as uncontrolled and long-term VEGF overexpression may have deleterious effects for ischemic myocardium [25]. It was reported that hypoxia response element promoter could regulate VEGF expression accurately and effectively in response to hypoxia and normoxia conditions [26]. For h-PAMAM-pHRE-hVEGF165 transfected SkMs, we observed that VEGF expression could be inhibited under normoxia but launched under hypoxia. Furthermore, the duration and level of VEGF expression were critical for achieving successful angiogenesis and recovery of heart function [27]. It was reported that VEGF overexpression for 1 to 2 weeks might be sufficient to form collateral vessels in ischemic myocardium [28]. In this study, the residual intracellular h-PAMAM-pHRE-hVEGF165 at 18 days after transfection may significantly contribute to long-term and stable hVEGF165 expression in vitro and in vivo.

The hypoxia-regulated VEGF overexpression in the graft region may improve angiogenesis, which reduced myocardial apoptosis, and increased graft SkM survival successfully. In our study, VEGF level in myocardium enhanced significantly during the 28 days after transplantation of SkMs-VEGF. However, it was observed that SkMs-0 transplantation also increased VEGF level. This effect may be related with the paracrine factors released by graft SkMs [29]. At one day after transplantation, cardiomyocyte apoptosis was reduced notably in the SkMs-VEGF grafted group. We speculate that this implantation strategy during the early infarction phase could provide cardioprotective effects through VEGF-induced vasodilatation as mediated by an increase in nitric oxide production that is independent of angiogenesis [30]. And our study also demonstrated that graft SkM survival improved significantly in Group 3. Improved graft survival could reinforce the cellular cardiomyoplasty effect as a result of an
improved blood supply to grafted myoblasts through both vasodilatation and enhanced angiogenesis. This would be particularly beneficial during the early stage after cell transplantation, when grafted cells are subjected to various pathological processes caused by environmental stresses, such as ischemia and mechanical injury [31]. Hence, we believe that VEGF overexpression could protect grafted cells from post-transplantation stress and improve their survival.

SkMs-VEGF transplantation was likely to reduce infarct size and interstitial fibrosis. After myocardial infarction, neovascularization is normally unable to compensate for the decreased blood supply and to support the tissue growth required for contractile compensation and the greater demands of the hypertrophied but viable myocardium. This may contribute to the death of viable myocardium, leading to progressive infarct size extension and interstitial fibrosis, which could be visualized by Masson trichrome staining [29]. In the present study, effective VEGF overexpression angiogenesis increased neovascularization in Group 3. The enhanced blood supply could contribute importantly to the reduction in infarct size and interstitial fibrosis, which could be observed clearly in the myocardium sections.

Heart function may well be improved eventually by application of the novel gene delivery system manipulated SkM transplantation. The results of echocardiography supported the evidence of improved heart function after the manipulated SkM transplantation. Though the insignificant difference between Group 2 and Group 3 could be found for LVedV, it may be related to the limited number of animals. Therefore, we confirmed that in the late phase, the VEGF induced angiogenic effect salvaging ischemic host myocardium, combined with cellular cardiomyoplasty effects in regenerating muscle could play an important role in improving heart function [32].

In summary, we developed h-PAMAM-pHRE-hVEGF165 as an economical, effective, biocompatible and controllable gene delivery system. The transplantation of h-PAMAM–pHRE-hVEGF165 manipulated SkMs could increase angiogenesis, inhibit left ventricle remodeling.
and improve heart function. This clinically relevant and combined strategy could be of importance for treating patients with acute myocardial infarction. And this novel gene delivery system may be applied in other ischemic disease in our future work.

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