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Novel hyperbranched polyamidoamine nanoparticles for transfecting skeletal myoblasts with vascular endothelial growth factor gene for cardiac repair

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Abstract We investigated the feasibility and efficacy of hyperbranched polyamidoamine (hPAMAM) mediated human vascular endothelial growth factor-165 ($hVEGF_{165}$) gene transfer into skeletal myoblasts for cardiac repair. The hPAMAM was synthesized using a modified one-pot method. Encapsulated DNA was protected by hPAMAM from degradation for over 120 min. The transfection efficiency of hPAMAM in myoblasts was 82.6 ± 7.0% with cell viability of 94.6 ± 1.4% under optimal conditions. The hPAMAM showed much higher transfection efficiency ($P < 0.05$) than polyetherimide and Lipofectamine 2000 with low cytotoxicity. The transfected skeletal myoblasts gave stable $hVEGF_{165}$ expression for 18 days. After transplantation of hPAMAM–$hVEGF_{165}$ transfected cells, apoptotic myocardial cells decreased at day 1 and heart function improved at day 28, with increased neovascularization ($P < 0.05$). These results indicate that hPAMAM-based gene delivery into myoblasts is feasible and effective and may serve as a novel and promising non-viral DNA vehicle for gene therapy in myocardial infarction.

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1 Introduction

Skeletal myoblast transplantation combined with gene therapy may be a potential approach to treating ischemic heart disease [1]. However, the gene vector still needs to be optimized. Traditional viral vectors enable high transfection efficiency but may cause inflammation and oncogenesis [2]. Polyamidoamine (PAMAM) dendrimers are a class of nanoscopic polymers emerging as suitable non-viral gene vehicles with low cell toxicity, high stability and encouraging transfection efficiency [3, 4]. However, the synthesis of PAMAM requires stepwise convergent approaches and repeated purification, and is extremely time-consuming and costly [5]. Hyperbranched polyamidoamine (hPAMAM), an analog to PAMAM, has similar chemical and physical properties to PAMAM but can be synthesized by an easier one-pot method [6]. To date, no studies have been reported that hPAMAM could be utilized in gene delivery systems. In the present study, we synthesized hPAMAM using a modified one-pot method, optimized conditions for transfection of hPAMAM nanoparticles into skeletal myoblasts, and investigated the feasibility and efficacy of this transfection protocol in the myocardial infarction animal models. We anticipate that hPAMAM mediated gene transfection will enable a new approach for gene therapy in cardiac repair.

2 Materials and methods

2.1 Synthesis of hyperbranched polyamidoamine nanoparticles

The synthesis of hPAMAM was carried out as described previously [7]. A solution of 20.6 g diethylene triamine

(DETA) in 25 ml of methanol (MA) was added to a one-neck flask and 20.6 g methyl acrylate was added dropwise into the reaction system. The mixture was then stirred at room temperature, and after 48 h the flask was fitted onto a rotary evaporator to remove the MA under the vacuum at room temperature. The mixture was then allowed to react under vacuum on the rotary evaporator for 1 h at 60°C, 1 h at 80°C, 1.5 h at 100°C, 1.5 h at 120°C, and 3 h at 140°C. The yellow product was precipitated in ethyl oxide three times and kept in a sealed container.

2.2 Preparation of hPAMAM–DNA complexes

Plasmids of enhanced green fluorescent protein (pEGFP) or human vascular endothelial growth factor-165 ($hVEGF_{165}$) (Gene Chem. Ltd., CHN) with hPAMAM nanoparticles were diluted separately in 50 μl of 150 mM NaCl. To determine the optimum ratio between hPAMAM and DNA during transfection, 14, 21, 28, 35 and 42 equivalents of hPAMAM nitrogen per DNA phosphate (N/P) were mixed to develop nanoparticle complexes. The mixture was vortexed gently, followed by sedimentation for 10 min, and then used freshly in the following experiments.

2.3 Characterizations of the hPAMAM–DNA complexes

The ultra shape of the complexes was visualized using transmission electron microscopy (TEM, JEM-1230, JEOL, JPN). Particle size distribution and zeta potential of hPAMAM–DNA complexes were detected with a Zetasizer instrument (Zetasizer Nano ZS, Malvern Instruments Ltd., UK). For determination of the resistance of hPAMAM–DNA complexes to nuclease digestion, 50 μl DNase I buffer (10×) was mixed with 450 μl solution containing 10 μg DNA or hPAMAM–DNA complexes. Then 2 U of DNase I (Fermentas, CAN) was added into the mixture and incubated at 37°C after 100 μl of solution was withdrawn at time 0. Samples (100 μl) were collected every 30 min up to 2 h during incubation. To halt DNase I degradation, all samples were treated with 10 μl ethylene diamine tetra-acetic acid (25 mM) for 10 min at 65°C. Finally, 10 μl sodium dodecyl sulfate (10%, w/v) was added into each sample and incubated for 24 h at 65°C in order to displace DNA from the hPAMAM nanoparticles. All samples were then placed in an ice bath and agarose gel electrophoresis was performed to evaluate the integrity of DNA bound to the nanoparticles.

2.4 Cell culture of C2C12

The C2C12 cells (cell line of skeletal myoblasts, donated from Chinese Academy of Science) were cultured and expanded in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) at 37°C under a humidified atmosphere containing 5% CO_2.

2.5 Optimization of hPAMAM–pEGFP transfection conditions

The transfection efficiency of hPAMAM–pEGFP was optimized and the in vitro cytotoxicity of hPAMAM–pEGFP evaluated as follows.

C2C12 cells were seeded at a density of 1×10^5/well in six-well plates and cultured for 24 h. Afterwards, hPAMAM–pEGFP complexes at different N/P ratios (14, 21, 28, 35 and 42) using 2 μg DNA were added into cells and the mixture was incubated at 37°C for 6 h. The transfection medium was then replaced with a fresh batch containing FBS. After 24 h, fluorescence images were photographed under a fluorescence microscope (LEICA DM IRE2, Leica, GER). Flow cytometry (LSR II SORP, BD, USA) was applied to evaluate the transfection efficiency. Nontransfected C2C12 and C2C12 transfected with hPAMAM nanoparticles without DNA were used for baseline setting of auto-fluorescence. Data were analyzed by WinMDI version 2.9 with gating at 1% for the optimum N/P ratio. To determine the optimum plasmid dosage during transfection, 2, 3, 4 and 5 μg DNA were transfected into cells by the same process as described above.

After cell transfection, the in vitro cytotoxicity of hPAMAM was evaluated by MTT [3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide blue-indicator dye]-based assay. C2C12 cells (1×10^5 cells/well) were placed into 96-well tissue-culture plates and incubated at 37°C. The hPAMAM–pEGFP complexes were then added into cells after 24 h at various N/P ratios (14, 21, 28, 35 and 42) and DNA dosages (2, 3, 4 and 5 μg). After 24 h, 20 μl of 5 mg MTT ml^{-1} solution was added into each well and the plate was incubated for another 4 h. The supernatant was removed and then Dimethyl sulfoxide was added to each well to dissolve the formazan. The results were read on a spectrophotometric microplate reader, using a wavelength of 570 nm. The following equation was used to calculate the inhibition of cell viability.

$$\text{Cell viability } (\%) = \mathrm{T}/\mathrm{C} \times 100\%$$

where C is the number of viable cells without nanoparticles and T is the number of viable cells after transfection.

2.6 Transfection efficiency and cytotoxicity of polyetherimide and Lipofectamine 2000

After combination with DNA according to the protocols, polyetherimide (PEI) and Lipofectamine 2000 (Sigma

Aldrich, USA) were transfected into skeletal myoblasts. The transfection efficiency and cytotoxicity of PEI and Lipofectamine 2000 were compared with those of hPAMAM nanoparticles.

2.7 In vitro skeletal myoblasts transfection with hPAMAM–hVEGF$_{165}$

Based on the results of flow cytometry and MTT, skeletal myoblasts were transfected with hPAMAM–hVEGF$_{165}$ complexes under the optimum transfection condition. The transfection and expression efficiency were analyzed by immunostaining, the reverse transcription polymerase chain reaction (RT-PCR), and the enzyme-linked immunosorbent assay (ELISA).

2.7.1 Immunostaining of skeletal myoblasts

The hPAMAM–hVEGF$_{165}$ complexes transfected skeletal myoblasts were seeded on chamber slides. The next day, samples were fixed with ice-cold ethanol. After incubation in 0.1% Triton X-100 and 1% bovine serum albumin, the samples were incubated in a 1:250 dilution of anti-human VEGF primary antibody (R&D, USA) at 4°C overnight, followed by incubation in a 1:50 dilution of horse radish peroxidase-conjugated secondary antibody. The hematoxylin was used for nuclear counterstaining and the immunostained cells were visualized by microscope.

2.7.2 RT-PCR analysis for hVEGF$_{165}$

Nontransfected, hVEGF$_{165}$ plasmid transfected, and hPAMAM–hVEGF$_{165}$ complexes transfected skeletal myoblasts were collected 2, 4, 8 and 18 days after transfection to quantify the VEGF expression (303 bp): Sense ATG AAC TTT CTG CTG TCT TGG, anti-sense GTT GGA CTC CTC AGT GGG C; 18 s was used as the internal control. After isolation of total RNA and cDNA synthesis, the QPCR thermal cycling program for 40 cycles was: 1 cycle of enzyme activation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 30 s.

2.7.3 ELISA for hVEGF$_{165}$

Supernatant samples from hPAMAM–hVEGF$_{165}$ transfected skeletal myoblasts were collected to quantify the VEGF expression with an ELISA kit (R&D, USA) at regular time intervals of 2 days (from day 0 until day 18) after transfection.

2.8 Cell transplantation in acute myocardial infraction animal models

The animal work was approved by the Institutional Review Board and Institutional Animal Care and Use Committee protocols of Fudan University.

Female C57/BL6 mice ($\approx$20 g) were anesthetized and mechanically ventilated. The myocardial infarction was created by means of permanent ligation of the left anterior descending coronary artery with an 8-0 polypropylene snare, 2 mm distal to the left auricle. Ten minutes later, DMEM without skeletal myoblasts (Group 1, $n = 10$) or containing 1×10^6 nontransfected skeletal myoblasts (Group 2, $n = 10$) or hPAMAM–hVEGF$_{165}$ transfected skeletal myoblasts (Group 3, $n = 10$) were intramyocardially injected into the infarct and peri-infarct regions.

2.9 In situ cell apoptotic assay

One day after cell transplantation, hearts ($n = 5$ animal each group) were harvested to detect apoptotic myocardial cells in the peri-infracted area using a TUNEL Detection Kit (Beyotime, CHN) according to the manufacturer's instructions. Tissue sections were counterstained with propidium iodide after TUNEL. The total number of cell nuclei and apoptotic nuclei were counted in five fields ($\times$200 magnification) per slide, and the percentage of apoptotic cardiomyocyte was calculated as the percentage of TUNEL positive nuclei to total nuclei per field.

2.10 Heart function assessment

At 4 weeks after cell transplantation, heart function of mice ($n = 5$ animals each group) was measured by an investigator blinded to the therapeutic intervention on the animals using an echocardiograph (Vevo770, VisualSonics, CAN). From M-mode echocardiogram, measurements were obtained for left ventricular internal diameters 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:

$$\mathrm{LVEF} = 1 - (\mathrm{LVIDes}/\mathrm{LVIDed})^2 \quad \text{and} \quad LVFS = 1 - (\mathrm{LVIDes}/\mathrm{LVIDed}).$$

2.11 Angiogenesis in peri-infarct area

After assessment of heart function, animal hearts (five animals in each group) were harvested. After perfusion, the

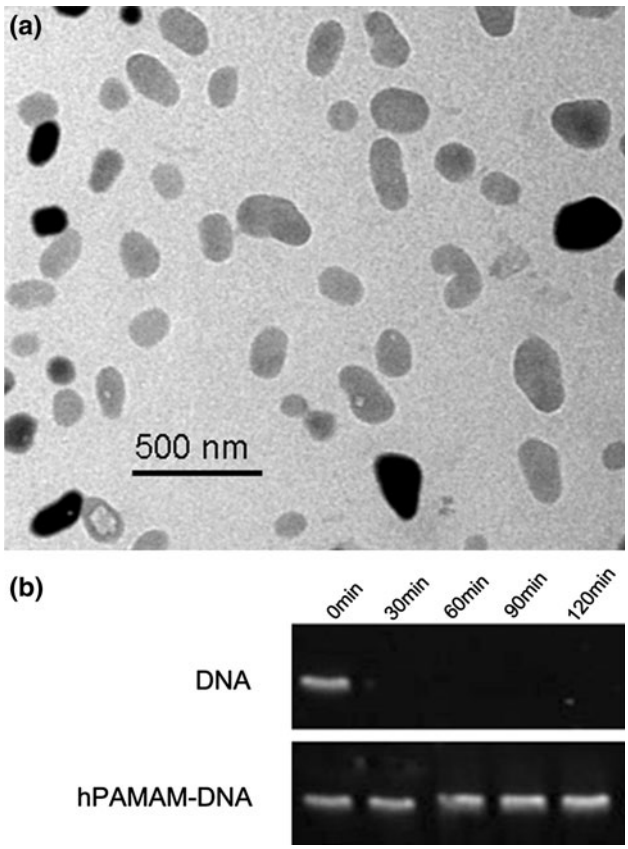


Fig. 1 **a** After combination with pEGFP plasmids, the ultra structure of nanoparticles appeared spheroidal (at N/P ratio of 28 using 3 μg DNA/1 × 10^5 cells). **b** The encapsulated DNA was protected by hPAMAM nanoparticles from DNase I digestion for over 120 min while the naked DNA was fully degraded in 30 min

left ventricle was sectioned into 6 μm segments parallel to the apex-base axis. The sections were immunostained with PECAM-1 (Santa Cruz, USA) for blood vessel density. During the process of counting the capillary vessel numbers, five sections around the infarction area were chosen in each mouse. For each section, five fields were chosen at ×400 magnification.

2.12 Statistical analysis

The data were analyzed with SPSS 17.0 software (SPSS, Inc). 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 Student's *t* test.

3 Results

3.1 Characterization of hPAMAM–pEGFP complexes

The molecular weight of hPAMAM was about 1.3 × 10^4 and the molecular weight polydispersity was 1.8. The weight of nitrogen per gram hPAMAM was 0.27 g.

After combination with pEGFP plasmids, the ultra structure of nanoparticles appeared spheroidal between 100 and 500 nm under TEM (Fig. 1a). The average particle sizes and zeta potential for various N/P ratios were shown in Table 1. The encapsulated DNA could be protected from degradation by hPAMAM for over 120 min, in contrast to the naked DNA, which was fully degraded by DNase I after 30 min (Fig. 1b).

3.2 Optimization of hPAMAM–pEGFP complexes transfection

The fluorescent spots could be observed in C2C12 cells with N/P ratios from 14 to 42 using 2 μg pEGFP plasmids/1 × 10^5 cells. Under ×200 magnification, the largest number of fluorescent spots appeared when N/P ratio was 28 (Fig. 2a). Similar results were also observed by flow cytometry. Maximum transfection efficiency (50.6 ± 2.0%), associated with low cell toxicity (cell viability = 94.3 ± 0.5%), was achieved when N/P ratio was 28 (Fig. 2b). With various DNA dosages from 2 to 5 μg at N/P ratio of 28, we found that higher transfection efficiency (82.6 ± 7.0%) with cell viability of 94.6 ± 1.4% could be achieved using 3 μg DNA/1 × 10^5 cells (Fig. 2c). Therefore, the optimum transfection condition was N/P ratio of 28 using 3 μg DNA/1 × 10^5 cells (Fig. 2d).

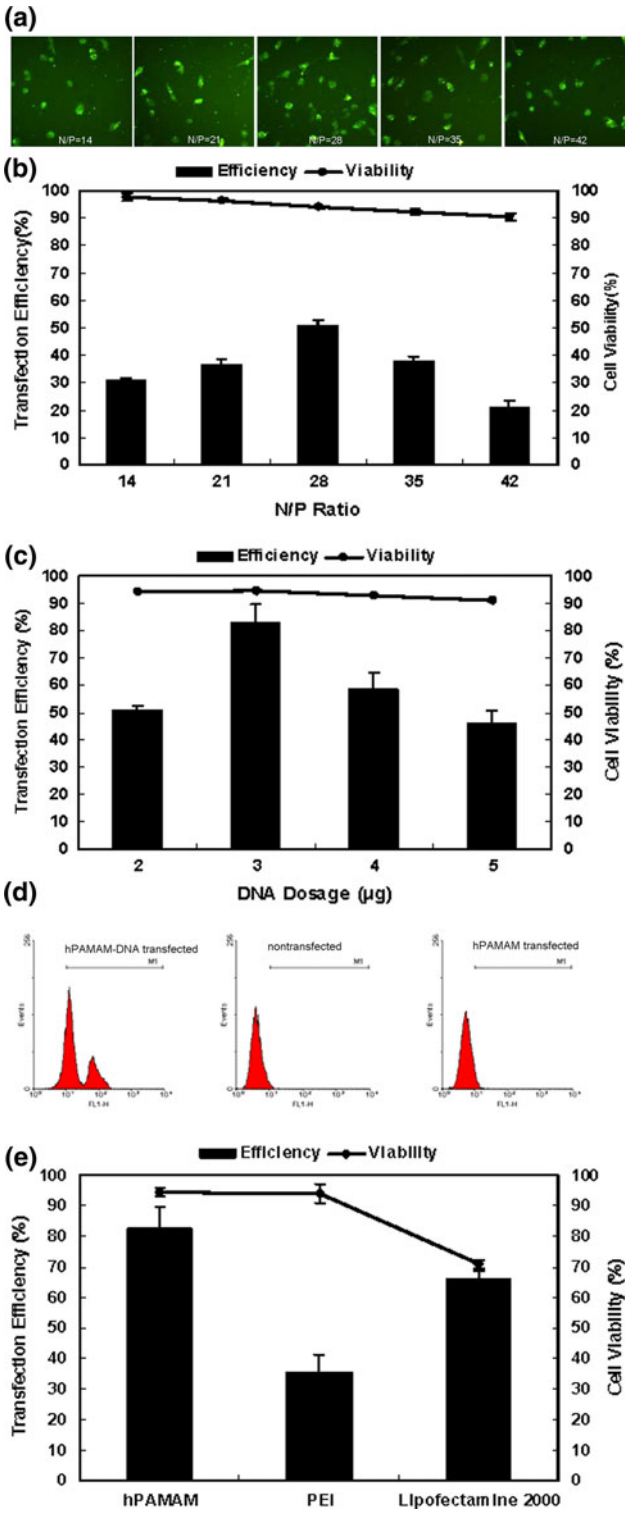
In C2C12 cells, hPAMAM could achieve higher transfection efficiency (82.6 ± 7.0%) than PEI and Lipofectamine 2000 (35.3 ± 5.9 and 65.9 ± 2.8% respectively, $P < 0.05$). The hPAMAM vector showed as high cell viability (94.6 ± 1.4%) as PEI (94.0 ± 3.1%), while cell viability in Lipofectamine 2000 was 70.9 ± 1.3% (Fig. 2e).

3.3 VEGF overexpression in the transfected C2C12 Cells

Immunostaining revealed hPAMAM–hVEGF$_{165}$ transfected cells could express hVEGF$_{165}$ protein in vitro (Fig. 3a). The RT-PCR showed gene expression increased 4.6 ± 0.2 times at day 2, 4.3 ± 0.2 times at day 4 and 2.5 ± 0.2 times at day 8 respectively, whereas it was 1.7 ± 0.1 times

Table 1 Particle size and zeta potential at various N/P ratios

N/P	41	21	28	35	42
Particle size (nm)	291.3 ± 5.4	369.9 ± 5.0	320.7 ± 4.6	308.7 ± 5.9	212.1 ± 5.4
Zeta potential (mV)	27.07 ± 1.36	30.27 ± 1.03	35.12 ± 176	36.72 ± 1.44	37.40 ± 2.71



◀**Fig. 2** **a** GFP expression in C2C12 cells at 24 h post-transfection with hPAMAM–DNA complexes at various N/P ratios (**a**–**e**: N/P = 14, 21, 28, 35 and 42, ×200 magnification). **b** Maximum gene transfection with optimum cell viability was achieved when N/P was 28 using 2 μg DNA/1 × 10^5 cells. **c** Transfection efficiency increased when N/P ratio was 28 using 3 μg DNA/1 × 10^5 cells. **d** Flow cytometry showed significantly higher GFP+ cells (82.6%) compared with nontransfected cells (0.7%) and hPAMAM transfected cells (1.6%) as controls. **e** Compared with PEI and Lipofectamine 2000, hPAMAM could achieve higher transfection efficiency under optimum transfection conditions with minor cytotoxicity

at day 18 (Fig. 3b, c). ELISA demonstrated that the transfected cells could provide stable $hVEGF_{165}$ protein secretion for 18 days stably, with the peak level of expression (791.7 ± 54.8 pg $hVEGF_{165}$ protein ml^{-1}) at day 2 after transfection (Fig. 3d).

3.4 Apoptosis

At 1 day after myocardial infarction, apoptosis in the peri-infarcted area was reduced in Group 2 (Fig. 4b) compared with Group 1 (Fig. 4a). This effect was further enhanced in Group 3 (Fig. 4c). The percentage of TUNEL positive cells (green) to total nuclei (red) in the peri-infarcted area was highest (19.0 ± 2.2%) in Group 1, whereas it was 11.6 ± 0.9% in Group 2 and 7.4 ± 1.1% in Group 3 (Fig. 4d).

3.5 Heart function studies

At 4 weeks after myocardial infarction, LVEF and LVFS in Group 1 were 33.9 ± 2.0 and 16.0 ± 1.0%, respectively. A significant improvement in LVEF and LVFS was observed in Group 2 (45.0 ± 3.5 and 22.2 ± 2.0%) and Group 3 (61.8 ± 5.6 and 29.4 ± 2.3%). Furthermore, LVEF and LVFS in Group 3 showed greater improvement than those in Group 2 ($P < 0.05$) (Fig. 5a, b).

3.6 Blood vessel density

Blood vessel density for PECAM-1 immunostaining (×400 magnification) was highest in Group 3 (77.9 ± 4.5) compared with Group 1 (24.6 ± 3.1) and Group 2 (44.4 ± 4.4) at 4 weeks after myocardial infarction ($P < 0.001$) (Fig. 6a–d).

4 Discussion

The hPAMAM, a structural analog to PAMAM dendrimers, has been proposed as an effective agent for gene delivery. Our present study modified the method of synthesis of hPAMAM, optimized transfection conditions of this novel gene vector and investigated the feasibility and efficacy of this gene transfection for cardiac repair.

In this study, we synthesized of hPAMAM by a modified one-pot method. In step 1, Michael addition of MA to DETA gave two kinds of intermediate at room temperature. The amidation reaction inevitably occurs between MA and DETA but Michael addition could react faster at the lower

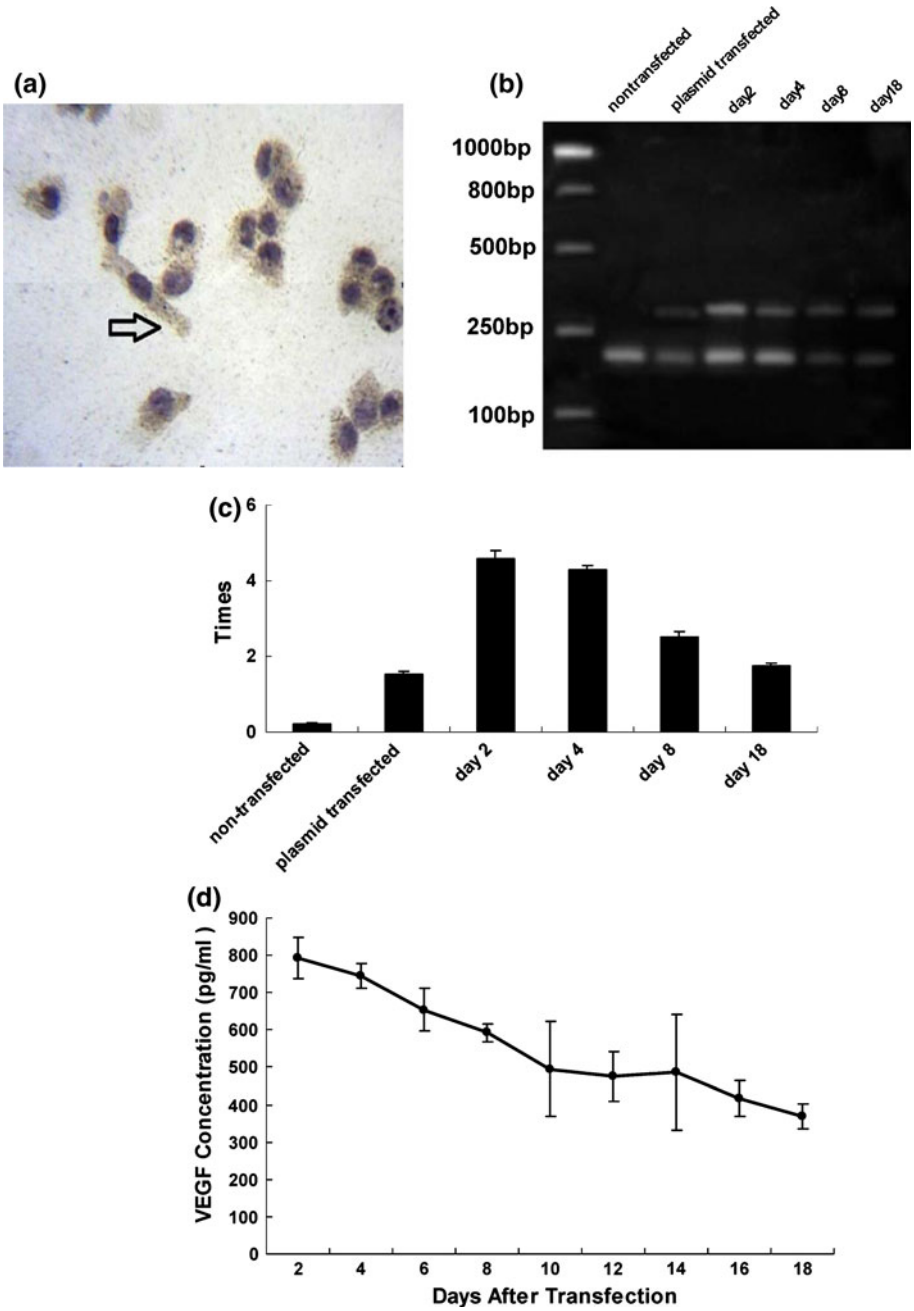


Fig. 3 **a** The $hVEGF_{165}$ expression (*arrowhead*) in hPAMAM–$hVEGF_{165}$ complexes transfected C2C12 cells (×200 magnification). **b** RT-PCR of hPAMAM–$hVEGF_{165}$ complexes transfected C2C12 at 2, 4, 8, and 18 days after transfection (DNA ladder: 100 bp). **c** Gene expression could achieve a maximum on day 2. **d** ELISA for VEGF expression from hPAMAM–$hVEGF_{165}$ complexes transfected C2C12 from 2 to 18 days

temperature [8]. We gradually increased the temperature to drive the intermediates to achieve hPAMAM by intermolecular reaction, including repeated Michael addition and amidation. As products of amidation and solvent, the MA was rapidly removed under vacuum and high temperature. Therefore, this step was carried out in rotary evaporator in our study. Also, in order to narrow the molecular weight distribution, we eliminated the untreated monomer and low molecular weight products via precipitation in diethyl ether step.

As a cell line of skeletal myoblasts, C2C12 cells are attractive donor cells because they have high proliferative rates in culture for up to 2 years [9]. Also, transplantation of C2C12 or C2C12 genetically engineered with angiogenic factor showed enhanced neovascularization without definite deleterious effect [10–12]. Therefore, we used C2C12 cell line as the donor cells.

In our present study, more importantly, we showed that the hPAMAM could be utilized as a potential gene vector with excellent protection for the plasmids, low cell toxicity, and high transfection efficiency in C2C12 skeletal myoblasts. Our study found that the hPAMAM–DNA complexes were resistant to digestion by DNase I for up to 120 min.

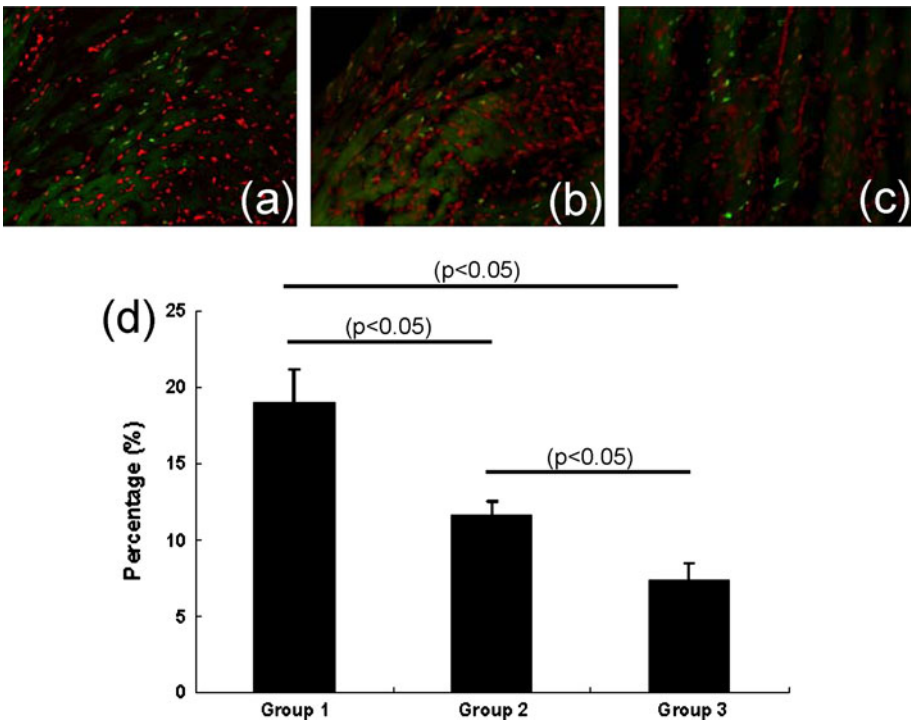


Fig. 4 Group 1 (**a**) had extensive apoptotic cells (*green*) compared with Group 2 (**b**) and Group 3 (**c**) (×200 magnification). **d** The percentage of apoptotic myocardial cells (*green*) to total nuclei (*red*) in Group 3 was lower than that in Group 1 and Group 2 ($P < 0.05$)

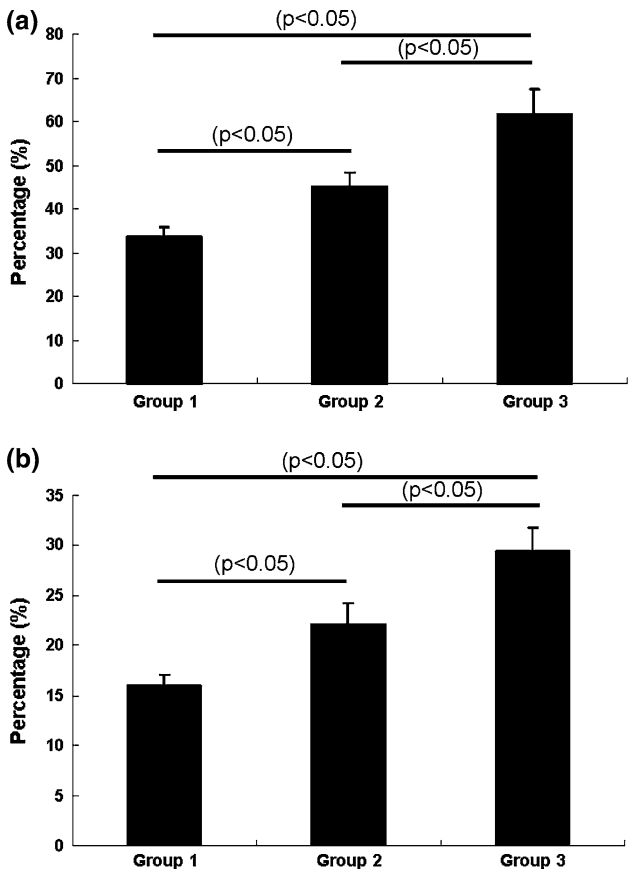


Fig. 5 LVEF (**a**) and LVFS (**b**) in Group 3 were significantly higher than those in Group 1 and Group 2 ($P < 0.05$)

This indicated that hPAMAM would render a significant protection to plasmids under physiological conditions, where the nuclease concentration is markedly lower than the tested concentration. In this study, hPAMAM also exhibited low cytotoxicity during transfection, which is a very important characteristic of gene vectors [13]. Our hPAMAM nanoparticles showed as excellent cell viability (94.6 ± 1.4% under optimum transfection conditions) as PEI (cell viability = 94.0 ± 3.1%), while Lipofectamine 2000 showed high cytotoxicity during transfection (cell viability = 70.9 ± 1.3%). Furthermore, hPAMAM showed amazingly high transfection efficiency in this study. As a structural analog, hPAMAM has similar chemical and physical properties to PAMAM, which often performs with encouraging efficiency during gene transfection. In a previous study, PAMAM could achieve higher transfection efficiency than PEI in 293 cells [14]. In 2009, Gu et al. [15] reported the transfection efficiency of PAMAM was 98% in breast cancer cells. More recently, using HEK293 cells, it was reported that the transfection efficiency of PAMAM was above 80% [16]. As a structural analog to PAMAM, against expectations, hPAMAM also showed transfection efficiency of 82.6 ± 7.0% under optimum conditions in C2C12 cells, which was much superior to that of PEI and Lipofectamine 2000 (transfection efficiency = 35.3 ± 5.9 and 65.9 ± 2.8%, respectively). This high transfection efficiency may be related to the large particle size (200–400 nm) and positive zeta potential, which is quite suitable for cell endocytosis [17, 18].

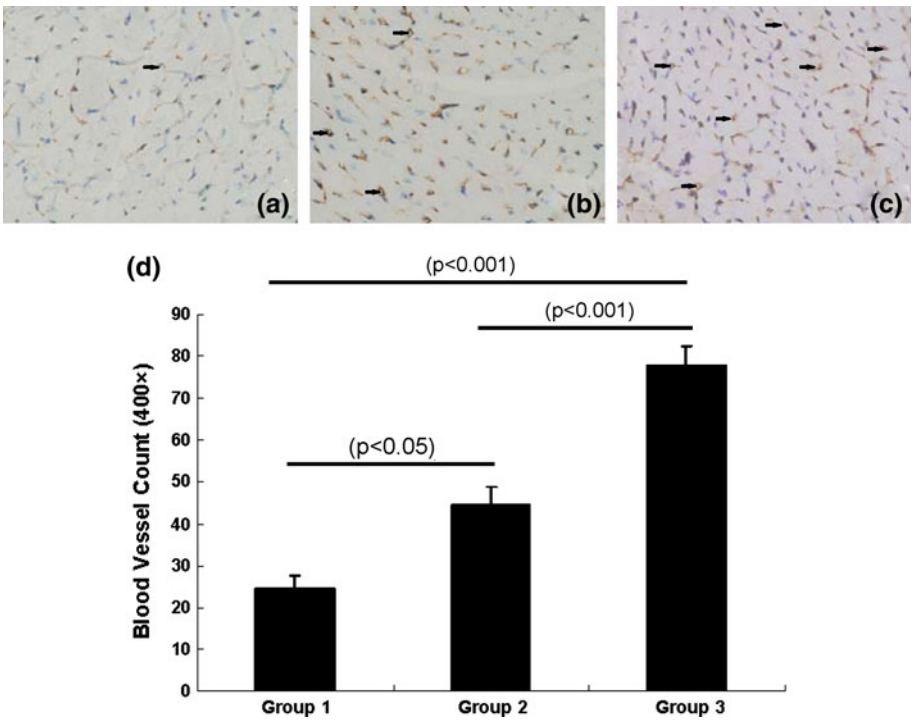


Fig. 6 Immunostaining for PECAM-1 in mice hearts of Group 1 (**a**), Group 2 (**b**) and Group 3 (**c**) at 4 weeks after cell transplantation (*arrowhead* for blood vessel, ×400 magnification). **d** Blood vessel count based on PECAM-1 was significantly increased in Group 3 compared with Group 1 and Group 2 ($P < 0.001$)

Using hPAMAM as a gene delivery vector to transfect skeletal myoblasts with $hVEGF_{165}$ plasmids, our study also showed good vascular endothelial growth factor (VEGF) expression in skeletal myoblasts, which later resulted in decreased myocyte apoptosis, increased blood vessel density, and increased heart functions. It was reported that transplantation of VEGF-expressing myoblasts could effectively treat ischemic heart diseases by VEGF-induced angiogenesis and skeletal myoblast-derived myogenesis [19, 20]. A more recent study reported that a period of 1–2 weeks of VEGF overexpression might be sufficient to induce collateral vessels in ischemic myocardium and it was sufficient to initiate neovascularization at a concentration of around 281.1 ± 62.6 pg $hVEGF_{165}/ml^{-1}$ after mesenchymal stem cell transplantation [21]. In our study, the transfected skeletal myoblasts gave stable overexpression of $hVEGF_{165}$ for 18 days. More importantly, the $hVEGF_{165}$ reached a peak concentration of 791.74 ± 54.80 pg protein/ml^{-1} on day 2 and maintained this for 18 days, with a concentration of 370.46 pg protein/ml^{-1} on day 18. Thus, we believe that the duration and magnitude of VEGF expression in our study was sufficient to initiate and maintain angiogenesis, which was in accordance with the increased blood vessel density and heart function improvement in our in vivo study.

5 Conclusion

In summary, we developed hPAMAM as a novel gene delivery vector with high transfection efficiency and low cytotoxicity. Transplantation of hPAMAM–$hVEGF_{165}$ transfected skeletal myoblasts could improve neovascularization and heart function. The hPAMAM-based nanoparticle gene delivery may enable a novel approach for gene therapy of ischemic heart disease.

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