

# An *in vitro* study of *pcDNA 3.0-hVEGF165* gene transfection in endothelial progenitor cells derived from peripheral blood of rabbits

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## Summary

The present study investigated the effect of the *VEGF165* gene on adhesion, migration, and proliferation of endothelial progenitor cells (EPCs) derived from peripheral blood of rabbits. Peripheral blood mononuclear cells were isolated from rabbits by density gradient centrifugation with Ficoll-Plaque Plus. EPCs were characterized by immunofluorescence and immunostaining. A *pcDNA 3.0-hVEGF165* expression vector was constructed and EPCs were transfected with the *pcDNA 3.0-hVEGF165* gene. The EPCs derived from peripheral blood of rabbits were successfully transfected with *pcDNA 3.0-hVEGF165*. ELISA showed that the expression of VEGF165 increased significantly in the EPCs transfected with the *hVEGF165* gene compared to control cells. Compared to control EPCs, EPCs transfected with the *hVEGF165* gene had significantly enhanced adhesion, migration, and proliferative ability *in vitro*.

**Keywords:** *hVEGF165* gene, endothelial progenitor cells, transfection, adhesion, migration, proliferation

## 1. Introduction

Asahara *et al.* (1) first reported the role of circulating endothelial progenitor cells (EPCs) in neovascularization and vasculogenesis in 1997. Over the past few decades, several studies have indicated that transplantation of EPCs effectively restored injured endothelia, repaired limb ischemia (2), myocardial ischemia (3), and carotid artery injury (4), and promoted vascular graft survival (5). A recent study by the current authors showed that transplantation of peripheral blood-derived EPCs effectively attenuated endotoxin-induced acute lung injury as a result of the paracrine effect of VEGF (6).

However, low concentrations of EPCs in peripheral blood precluded the extensive use of EPCs. A study indicated that VEGF stimulates the proliferation, migration, and survival of endothelial cells, in turn

facilitating endothelialization with EPCs and recovery of endothelial function (7). VEGF was first discovered by Ferrara (8) and is a glucoprotein isolated from cattle pituitary gland follicle stellate cells. VEGF helps maintain vessel normal shape and integrity, promote vessel permeability, and facilitate endothelial cell proliferation and vessel production (9). Five different VEGF protein isoforms, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E, have been derived from a single gene by alternative splicing (10). There are five characterized VEGF-A isomers of 121, 145, 165, 189, and 206 amino acids in mammals that are generated by alternative splicing of the mRNA from a single gene consisting of eight exons. VEGF165 is the most powerful function protein that promotes angiogenesis in the VEGF family. However, its short half-life and few natural sources mean that an effective concentration of VEGF165 cannot be maintained in the blood after injection. The use of *ex vivo*-transfected stem cells as vectors for gene therapy has largely solved this problem. A study reported that transplantation of VEGF-transfected EPCs increased vasculogenesis and required fewer EPCs than non-transfected EPCs (11). Moreover, the role of VEGF genes transfected

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in EPCs has yet to be fully elucidated. Therefore, the current study sought to investigate the effects of EPCs transfected with the *hVEGF165* gene on the adhesion, migration, and proliferative ability of EPCs *in vitro*.

## 2. Materials and Methods

### 2.1. Materials

New Zealand White rabbits were obtained from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. Ficoll-Plaque Plus was purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). EGM-2 MV Single Quots were obtained from Lonza Corp (Basel, Switzerland). DiI-acetyl-low density lipoprotein was purchased from Invitrogen (Carlsbad, CA, USA). Fluorescein isothiocyanate-ulex europaeus agglutinin-1 (UEA-1) was from Sigma-Aldrich (St. Louis, MO, USA). VEGFR2 and CD133 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A hVEGF165 ELISA detection kit was provided by Chemicon International Inc., Temecula, CA, USA.

### 2.2. Isolation and culture of peripheral blood EPCs

Approval was obtained from the Animal Care and Use Committee of Hospital No. 455 of the PLA. Peripheral blood was obtained from an ear artery of New Zealand White rabbits (10 mL/kg). Peripheral blood mononuclear cells were isolated by density gradient centrifugation with Ficoll-Plaque Plus. Mononuclear cells were then washed and plated on six-well plates supplemented with EGM-2 MV Single Quots. Mononuclear cells were incubated at 37°C, 5% CO<sub>2</sub> and supplied daily with EGM-2. The nonadherent cells were removed after 48 h of culture. On day 7 of culturing, the adherent cells (known as early EPCs) were detached with 0.025% trypsin containing 0.02% EDTA and used for analysis or transplantation.

### 2.3. Characterization of EPCs

The EPCs isolated from rabbits were characterized as previously described (8). Cells were incubated with 10 µg/mL DiI-acetyl-low density lipoprotein (LDL) and 5 µg/mL fluorescein isothiocyanate-*Ulex europaeus* agglutinin-1 (UEA-1). The staining of acetyl-LDL and UEA-1 in cultured EPCs was detected under fluorescence confocal microscopy at the absorption wavelengths of 555 and 490 nm, respectively. Dual fluorescent staining positive for both fluorescein isothiocyanate-labeled UEA-1 and DiI-labeled acetyl-LDL (double-positive cells) served to identify differentiating EPCs. Immunostaining of vascular endothelial growth factor receptor 2 and CD133 was performed as previously described.

### 2.4. Construction and identification of a pcDNA 3.0-hVEGF165 expression vector

The recombinant retroviral vectors pLXSN-KDRp-VEGF165 and pcDNA 3.0 vectors were constructed in this laboratory. PLXSN-KDRp-VEGF165 was digested by Bam HI and XhoI to release KDRp-VEGF165. The KDRp-VEGF165 fragment was ligated with Bam HI and XhoI digested pcDNA 3.0 to form a pcDNA 3.0-KDRp-VEGF165 recombinant vector. Then, pcDNA 3.0-KDRp-VEGF165 was digested with EcoR I to remove KDRp and self-ligated to generate a pcDNA 3.0-VEGF165 recombinant plasmid. pcDNA 3.0-VEGF165 was verified by XhoI I and Hind III digestion and sequencing analysis.

### 2.5. Transfection of EPCs with the pcDNA 3.0-hVEGF165 expression vector

After EPCs were cultured for 7 days according to the manufacturer's instructions in the DOTAP Liposomal kit, EPCs were transfected with pcDNA 3.0-hVEGF165 and eGFP (group A). EPCs transfected with null-plasmid (group B) and nontransfected EPCs (group C) served as controls. After 24 h, the EPCs were detected using fluorescent microscopy.

### 2.6. ELISA detection of VEGF165 expression *in vitro*

pcDNA 3.0-hVEGF165-transfected EPCs were grown in six-well plates at a cell density of  $1 \times 10^5$  cells per well, using EPCs transfected with null-plasmid and nontransfected MSCs as controls. The supernatant were harvested at two-day intervals from day 1 to day 7 post-transfection. The samples were centrifuged and then stored at -70°C until the assay. A human VEGF165 ELISA kit was used to detect the VEGF165 protein secreted from transfected EPCs in accordance with the manufacturer's instructions.

### 2.7. Detection of the adhesive ability of EPCs

pcDNA 3.0-hVEGF165-transfected EPCs and null-plasmid and nontransfected EPCs were washed with PBS and gently detached with 0.25% trypsin. After centrifugation and resuspension in EGM-2, identical cell numbers were replated onto fibronectin-coated culture dishes and incubated for 30 min at 37°C. Adherent cells were counted.

### 2.8. Migration assay

EPC migration was evaluated using a transwell chamber assay. In brief, the pcDNA 3.0-hVEGF165-transfected EPCs and null-plasmid and nontransfected EPCs were detached with 0.25% trypsin, and then  $1 \times 10^5$  EPCs in 200 µL EGM-2 were seeded in the upper chamber of a

transwell cell culture insert (5  $\mu\text{m}$  pore size). VEGF (50 ng/mL, PeproTech, Rocky Hill, NJ, USA) in EGM-2 was placed in the lower chamber. After incubation for 6 h at 37°C, the membranes were washed with phosphate buffer solution (PBS) and fixed with methanol. EPCs were stained with 4,6-diamidino-2-phenylindole (DAPI) solution. Light microscopy (400-fold magnification) was used to count the numbers of EPCs that had migrated to the lower surface of the membrane from five random fields in five domains.

### 2.9. MTT assay of EPC proliferation

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to determine EPC proliferation. pcDNA 3.0-hVEGF165-transfected EPCs and null-plasmid and nontransfected EPCs were digested with 0.25% trypsin and then cultured in 96-well plates (200  $\mu\text{L}$ /well). EPCs were supplemented with 10  $\mu\text{L}$  MTT (5 g/L, Sigma-Aldrich) and incubated for another 4h. The supernatant was then discarded by aspiration and the EPC preparation was shaken with 150  $\mu\text{L}$  dimethyl sulfoxide (DMSO) for 10 min before the OD value was measured at 490 nm.

### 2.10. Statistical analysis

All data are presented as mean  $\pm$  S.D. Parametric data were analyzed using one-way ANOVA and variations in different groups were compared using the Turkey *post hoc* test.  $p < 0.05$  was considered statistically significance.

## 3. Results

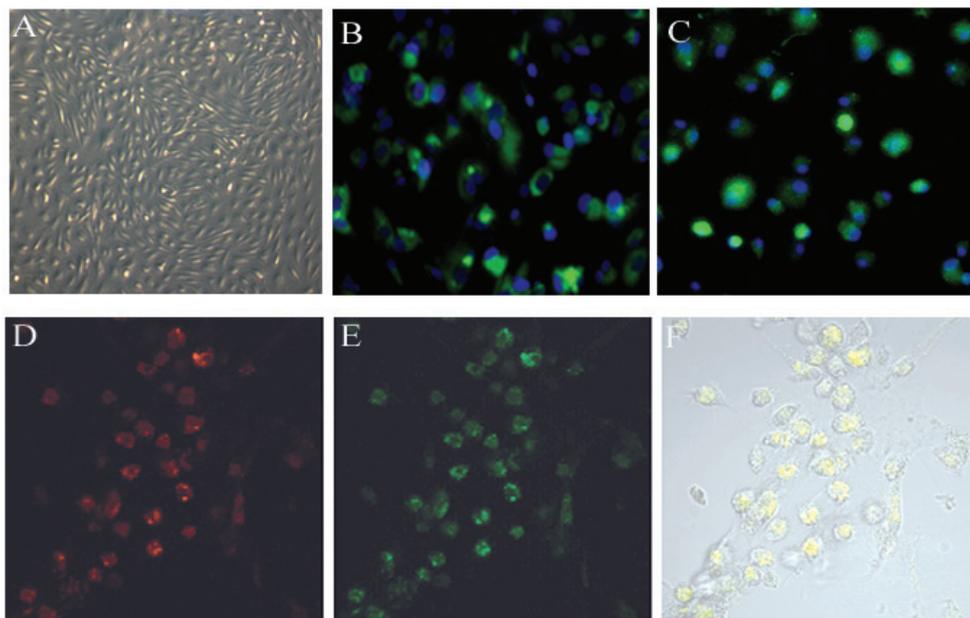
### 3.1. Characterization of EPCs

Seven days after cultivation, EPCs (approximately  $2-3 \times 10^6$  cells) proliferated from 20 mL peripheral blood of each rabbit. Formation of monolayer colonies with a "cobblestone" appearance was also observed after 2 weeks of culturing in endothelial basic medium 2 (Figure 1A). The EPCs also displayed expression of endothelial markers vascular endothelial growth factor receptor 2 (Figure 1B) and CD133 (Figure 1C). Fluorescence confocal microscopy showed that these early EPCs exhibited phenotyping of endothelial cells, including incorporation of DiI-ac-LDL and binding of fluorescein isothiocyanate-UEA-1. Dual fluorescent staining positive for both fluorescein isothiocyanate labeled UEA-1 and DiI-ac-LDL (double-positive cell) served to identify differentiating EPCs (Figures 1D-1F).

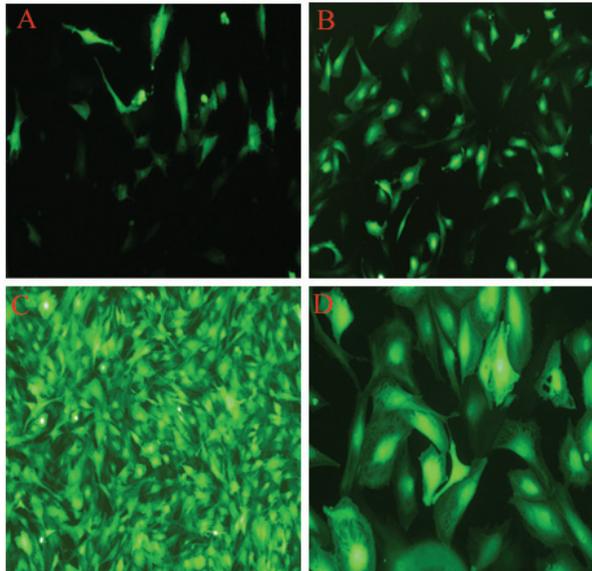
### 3.2. Identification of the pcDNA 3.0-hVEGF165 expression vector

#### 3.2.1. Fluorescence detection of hVEGF165-eGFP-transfected EPCs

In inverted fluorescence microscopy, EPCs had little green fluorescence 1 day after transfection with hVEGF165-eGFP (Figure 2A). Forty-eight h after transfection with hVEGF165-eGFP, EPCs fluoresced green (Figure 2B). Moreover, after day 3 hVEGF165-eGFP-transfected EPCs had substantial green



**Figure 1. Characterization of EPCs.** Cultured for 14 days, EPCs grew into monolayer with a "cobblestone" appearance (A, 100 $\times$  magnification). Immunofluorescent EPCs were positive for cytoplasmic vascular endothelial growth factor receptor 2 and CD133 (B, 200 $\times$  magnification, and C, 200 $\times$  magnification, green fluorescence). Nuclei were counterstained in blue fluorescence. Fluorescence confocal microscopy (200 $\times$  magnification) revealed that after day 7 the endothelial progenitors were positive for the uptake of DiI-acLDL (D, red fluorescence) and for staining of FITC-UEA-1 (E, green fluorescence). D and E results are superimposed in panel F (yellow fluorescence).



**Figure 2. Fluorescence detection of hVEGF165-eGFP-transfected EPCs.** Fluorescence microscopy revealed little green fluorescence 1 day after EPCs were transfected with hVEGF165-eGFP (A, 200× magnification). Two days after transfection with hVEGF165-eGFP, EPCs fluoresced green (B, 200× magnification). Moreover, after day 3 hVEGF165-eGFP-transfected EPCs had substantial green fluorescence (C, 200× magnification; D, 400× magnification).

fluorescence (Figures 2C and 2D).

### 3.2.2. VEGF165 protein levels in EPCs

VEGF protein expression was confirmed by ELISA. As shown in Figure 3, the VEGF protein level in group A was higher than that in group B ( $p < 0.05$ ) and group C ( $p < 0.05$ ). Moreover, the level increased significantly after 3 days, reaching 1,255 pg/mL after 7 days.

### 3.3. Assay of EPC adhesion

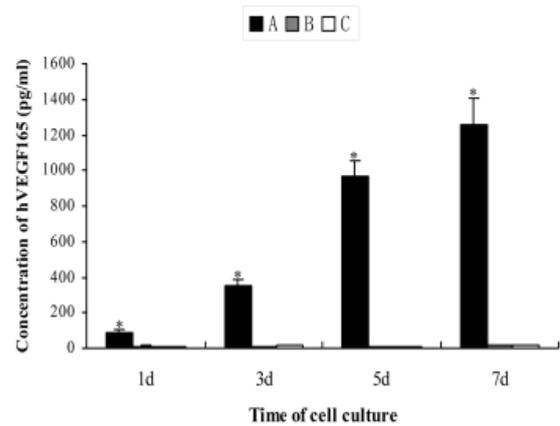
To investigate the possibility that pcDNA 3.0-hVEGF165-transfected EPCs alter the adhesion of EPCs, an adhesion assay was performed. EPC adhesion was significantly enhanced in the pcDNA 3.0-hVEGF165-transfected EPCs ( $66.2 \pm 6.4$ , Figure 4) compared to the null-plasmid-transfected EPCs ( $33.5 \pm 4.4$ ) and nontransfected EPCs ( $35.7 \pm 6.3$ ).

### 3.4. Assay of EPC migration

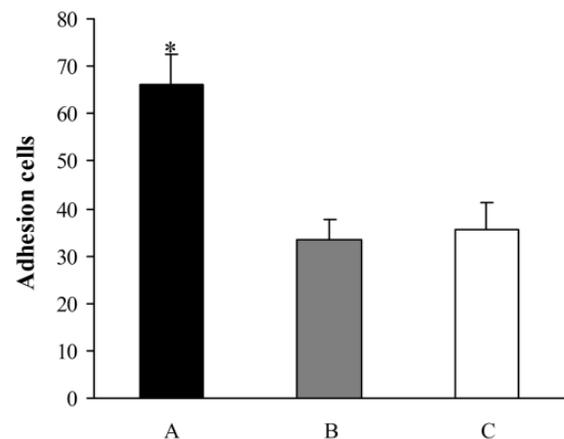
The influence of VEGF165-transfected EPC migration was analyzed in a transwell chamber assay. As shown in Figure 5, the migration of EPCs was significantly enhanced in group A ( $43.6 \pm 2.5$ ) compared to group B ( $19.8 \pm 2.8$ ) and group C ( $17.6 \pm 3.2$ ) ( $p < 0.01$ ).

### 3.5. Assay of EPC proliferation

The MTT assay was used to detect the proliferative activity of transfected EPCs. The *in vitro* proliferative



**Figure 3. ELISA detection of VEGF165 protein levels in EPCs.** VEGF protein expression was detected with ELISA. VEGF protein level in group A was higher than that in group B and group C. Moreover, the VEGF protein level increased significantly ( $*p < 0.05$ , A vs. B and C;  $n = 5$  different samples in each group) after 3 days, reaching 1,255 pg/mL after 7 days. Data are presented as mean  $\pm$  S.D. Statistical analysis was performed using ANOVA followed by the Turkey multiple comparison test.

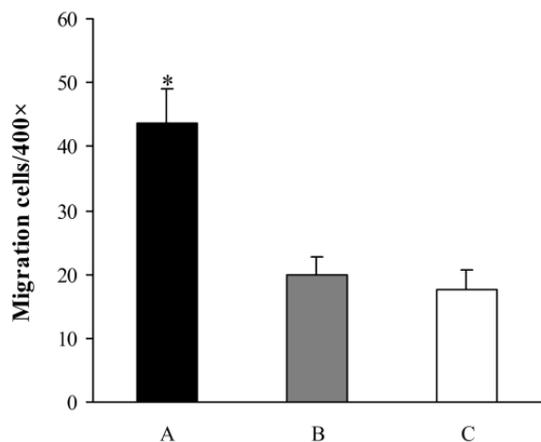


**Figure 4. Assay of EPC adhesion.** To investigate the possibility that the pcDNA 3.0-hVEGF165-transfected EPCs alters the adhesion of EPCs, an adhesion assay was performed. Compared with the null-plasmid transfected EPCs and nontransfected EPCs, EPC adhesion was significantly enhanced ( $*p < 0.05$ , A vs. B and C;  $n = 5$  different samples in each group) in the pcDNA 3.0-hVEGF165-transfected EPCs. Data are presented as mean  $\pm$  S.D. Statistical analysis was performed using ANOVA followed by the Turkey multiple comparison tests.

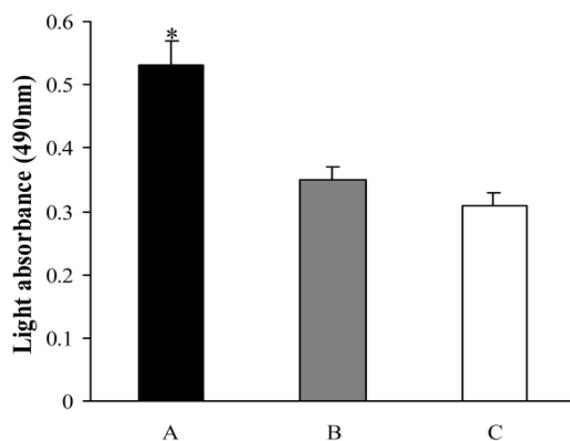
activity of the pcDNA 3.0-hVEGF165-transfected EPCs ( $0.53 \pm 0.04$ ) was greater than that of the null-plasmid-transfected EPCs ( $0.35 \pm 0.02$ ) and nontransfected EPCs ( $0.31 \pm 0.02$ ) ( $p < 0.05$ ). The proliferative activity of null-plasmid-transfected EPCs was similar to that of nontransfected EPCs (Figure 6).

## 4. Discussion

In the present study, EPCs derived from peripheral blood of rabbits were successfully transfected with the pcDNA 3.0-hVEGF165 gene. The expression of



**Figure 5. Assay of EPC migration.** The influence of VEGF165-transfected EPC migration was analyzed in a transwell chamber assay. Using light microscopy (400-fold magnification), the numbers of EPCs that had migrated to the lower surface of the membrane were counted in five random fields in five domains. EPC migration was significantly enhanced ( $*p < 0.05$ , A vs. B and C;  $n = 5$  different fields in each group) in group A compared to group B and group C. Data are presented as mean  $\pm$  S.D. Statistical analysis was performed using ANOVA followed by the Turkey multiple comparison test.



**Figure 6. MTT assay of EPC proliferation.** The MTT assay was used to detect the proliferative activity of transfected EPCs. The *in vitro* proliferative activity of pcDNA 3.0-hVEGF165-transfected EPCs was greater than that of null-plasmid transfected EPCs and nontransfected EPCs. The proliferative activity of null-plasmid transfected EPCs was similar to that of nontransfected EPCs ( $*p < 0.05$ , A vs. B and C;  $n = 6$  different samples in each group). Data are presented as mean  $\pm$  S.D. Statistical analysis was performed using ANOVA followed by the Turkey multiple comparison test.

VEGF165 increased significantly in EPCs transfected with the *hVEGF165* gene. Moreover, EPCs transfected with the *hVEGF165* gene had significantly enhanced adhesion, migration, and proliferative ability.

Previous studies indicated that EPCs harvested from the peripheral circulation proliferated *ex vivo* and EPCs transplanted *in vivo* play an important role in angiogenesis and repair of injured endothelium (12-14). More recent findings suggest that transplantation of circulating EPCs into an injured vascular wall has

beneficial effects on vascular structure and function (15-18). Transplantation of EPCs derived from peripheral blood thus constitutes a novel therapeutic strategy that could provide a robust source of viable endothelial cells to supplement the contribution of endothelial cells. However, healthy adults only have 2-3/mL of EPCs in peripheral blood. Patients with diabetes who are receiving a transplantation of EPCs have fewer, dysfunctional, and rapidly aging EPCs. Although culture and amplification of EPCs *ex vivo* can increase the quantity and purity of EPCs, EPC dysfunction in the peripheral blood restricted the extensive use of EPCs. Therefore, an important challenge was to provide EPCs of sufficient quantity and function for transplantation.

Gene transfer of EPCs during *in vitro* expansion could be used to achieve phenotypic modulation of EPCs. Furthermore, phenotypic modulation of EPCs might also reduce the number of EPCs required for optimal transplantation after *in vitro* expansion and thus serve to address a practical limitation of EPC transplantation, namely the volume of blood required to extract an optimal number of EPCs for autologous transplantation. A study indicated that VEGF stimulates proliferation, migration, and survival of endothelial cells, in turn facilitating endothelialization with EPCs and recovery of endothelial function (7). A recent study found that EPCs produced and secreted more proangiogenic cytokines, such as VEGF, promoting angiogenesis (6). Because of its short half-life, however, an effective concentration of VEGF cannot be maintained in the blood after injection. The use of *in vitro*-transfected stem cells as vectors for gene therapy has largely solved this problem. A study reported that transplantation of VEGF gene-modified EPCs reduced limb ischemia and improved neovascularization compared to control EPCs. The dose of VEGF-transfected EPCs transplanted was 30 times lower than that of nontransfected EPCs (11).

The current findings showed that hVEGF165 gene-modified EPCs increased the levels of VEGF protein and enhanced the ability of EPCs to adhere, migrate, and proliferate. The results of *in vitro* studies provided potential insights into the mechanisms responsible for the *in vivo* outcomes. First, EPCs transfected with the hVEGF165 may increase overexpression of VEGF, which has paracrine effects that promote the restoration of injured endothelial cells. Previous studies demonstrated that VEGF induced upregulation of certain endothelial cell integrins and matrix proteins (19). Second, EPCs transfected with the *hVEGF165* gene enhance the adhesion, migration, and proliferation of EPCs in the quiescent endothelial cell monolayer, and the survival of EPCs is probably due to the reduced requirement for harvested EPCs. Therefore, EPCs likely operate as vector stem cells, promoting local overexpression of VEGF that may in

turn promote migration, proliferation, and remodeling of differentiated endothelial cells residing in the target tissue.

In conclusion, *in vitro*, EPCs were successfully transfected with the *hVEGF165* gene. The expression of VEGF165 increased and adhesion, migration, and proliferative ability were significantly enhanced in EPCs transfected with the *hVEGF165* gene.

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