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Transfection of bone marrow mesenchymal stem cells using green fluorescence protein labeled hVEGF165 recombinant plasmid mediated by liposome

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ABSTRACT

Objective: To study the role of bone marrow mesenchymal stem cells (BMSCs) in construction of vascularized engineered tissue. **Methods:** hVEGF165 was amplified via RT-PCR before recombinant with pShuttle- green fluorescence protein;green fluorescent protein (GFP)-CMV. Then the recombinant shuttle plasmid was transfected into BMSCs with Lipofectamine™ 2000 for packaging and amplifying. hVEGF165 mRNA expression in BMSCs cells was tested. **Results:** The sequence of hVEGF165 in pShuttle-GFP-hVEGF165 plasmid was confirmed by double-enzyme cleavage method and sequencing. hVEGF165 was highly expressed in BMSCs. **Conclusions:** The GFP/hVEGF165 recombinant plasmid vector was constructed successfully and expressed effectively in host cells, which may be helpful for discussing the possibility of the application of VEGF165-BMSCs in tissue engineering and ischemic disease cure.

1. Introduction

In recent years, the application of vascular endothelial growth factor (VEGF) gene therapy such as acute myocardial infarction and chronic myocardial ischemia, peripheral artery disease, gastric duodenal ulcer and other diseases, has become a hot research at home and abroad[1]. Green fluorescent protein (GFP) is a protein which comes from the jellyfish, and under certain excitation light shows green fluorescent[2]. Bone marrow mesenchymal stem cells (BMSCs) is a kind of adult stem cells, which has been widely used and investigated in both tissue engineering and the treatment of various diseases. This study was to discuss the role of bone marrow mesenchymal stem cells (BMSCs) in construction of vascularized engineered tissue.

2. Materials and methods

2.1. Experiment instruments and reagents

PCR (Bio-Rad, USA); BIO-RAD Gel Doc XR + Gel imaging system (BIO-RAD, USA). α-minimal essential medium (Hyclone, USA), 0.25% Trypsin-EDTA (Gibco, USA); ShengGong primer synthesis (Shanghai); Total RNA extraction reagent (TaKaRa Biotechnology, Dalian); Lipofectamine transfection™ 2000 kit (Invitrogen, USA); PrimeScript® RT reagent Kit With gDNA Eraser (TaKaRa Biotechnology, Dalian); DNA Marker (TaKaRa Biotechnology, Dalian); 2×Pfu PCR Master Mix (TIANGEN technology co., LTD., Beijing, China); PShuttle GFP-CMV (in this lab, preliminary build) AdEasy-1 system (Stratagene, USA), competent *Escherichia coli* (*E. coli*) DH5, restriction enzyme *Bam*HⅠ, *Xho*Ⅰ, *Hand* Ⅲ, *Eco*RⅠ and standard

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DNA marker (Promega); VEGF antibody ((Bioss, China); DAB chromogenic reagent kit (Dako, USA).

2.2. Methods

2.2.1. Construction and identification of recombinant shuttle plasmid vectors

hVEGF165 was amplified from pcDNA3. 1-VEGF165 plasmid (sequenced to be correct), followed by agarose gel electrophoresis and purification. *Bam*HⅠ and *Xho* Ⅰ enzyme were used for recycling of PCR products and pShuttle GFP-double enzyme CMV, electrophoresis was performed with plastic recycling kit after purification at 16 ℃ by T4 DNA ligase connection over night. pShuttle GFP-hVEGF165 recombinant plasmid was obtained. *E. coli* DH5 alpha cells was transformed by calcium chloride method, and recombinant plasmid was screened by blue white spot screening. Plasmid was extracted by alkali decomposition method. Then it underwent double enzyme digestion by *Bam*HⅠ and *Xho* Ⅰ and sequencing identification.

2.2.2. pAd-GFP-building and identification of hVEGF165 recombinant plasmid

pShuttle GFP-hVEGF165 recombinant plasmid was digested and linearized by *Pme* Ⅰ. pAdEasy-1 was transformed into the *E. coli* by hot shock method, and screened by Amp + resistance screening. BJ5183 competent bacterium (including pAdEasy-1) was transformed by electronic shock with 1 μg linearized pShuttle GFP-hVEGF165 plasmid (2.2 kV, 2.2 ms), and then was screened by kanamycin resistance screening. Plasmid was extracted by alkali decomposition method, identified by *Bam*HⅠ, *Pac* Ⅰ, respectively. Recombinant plasmid of pAd-GFP-hVEGF165, VEGF specific fragments were amplified by PCR, hVEGF165 gene sequence was detected, and positive clones were amplified largely.

2.2.3. Transfection of VEGF165 recombinant plasmid

With *Pac* Ⅰ enzyme, pAd-GFP-hVEGF165 was digested and linearized, then was transfection into HEK 293T cells. Recombinant plasmid and empty vector plasmid were extracted, then was transfection into HEK 293T cells according to Lipofectamine™ 2000 instructions. The expression of green fluorescent protein was observed at 24 h and 6 day.

2.2.4. Construction and identification of BMSCs

Upper and lower end of metaphysis was removed in adult rat, and bone marrow was mixed with α-MEM containing 20% fetal bovine serum in petri dishes. It was blew and beat by a straw to make cells scattered and was incubated at 37 ℃, 5% CO_2 and saturated humidity for normal pancreatic enzyme digestion. The 4th generation cells of BMSCs was taken and fixed with 4% paraformaldehyde for 2 h. It was washed by PBS, added with 3% H_2O_2 at room temperature for 20 min to inactivate endogenous peroxidase, then it was washed again with PBS. It was blocked by goat serum for 1 h, with rat anti VEGF antibody as first antibody, biotin labeled goat anti mouse antibody as the second antibody. SH3 expression in transfected cells was detected by DAB chromogenic method.

2.2.5. Transfection of BMSCs by lipofection

According to Lipofectamine™ 2000 liposome transfection reagent instruction, 4th generation cells of BMSCs were digested by pancreatic enzyme, and then was counted. It was inoculated at 3×10^4/mL to 24 hole plate. When fusion of cell growth was around 80%, plasmid DNA of pAd-GFP-hVEGF165 diluted by OptiMEM® Ⅰ Medium and Lipofectamine™ 2000 diluted by OptiMEM® Medium was mixed, and incubated for 20 min; Mixture was added to each hole, and incubated at 37 ℃ and 5% CO_2, saturated humidity. After 6 h it was cultured by DMEM containing 10% FBS/F-12 media, and then was screened and cultured.

2.2.6. RT-PCR for detecting hVEGF gene expression after transfection

Total RNA of BMSCs was extracted after transfection, cDNA was obtained by reverse transcription. According to the instruction manual, 2×Pfu PCR Master Mix was used to detect the expression of VEGF mRNA. Forward primer: 5′- GGAGGGCAGAATCATCACGAAG-3′, reverse primer: 5′-CACACAGGATGGCTTGAAGATG-3′. Amplification length was 138 bp. Forward primer of β-actin: 5′-ACGGTCAGGTCATCACTATCG-3′, reverse primer: 5′-GGCATAGAGGTCTTTACGGATG-3′, amplification length was 155 bp. Image was obtained by BIO-RAD Gel Doc XR+ Gel imaging system. Transfected VEGF cells were identified by immunohistochemical assay.

3. Results

3.1. PCR identification of recombinant shuttle plasmid vector

Recombinant shuttle plasmid product was transformed into *E. coli* DH5 alpha, single colony was selected as a template to test strain. The results revealed hVEGF165 expression vector in same size (Figure 1).

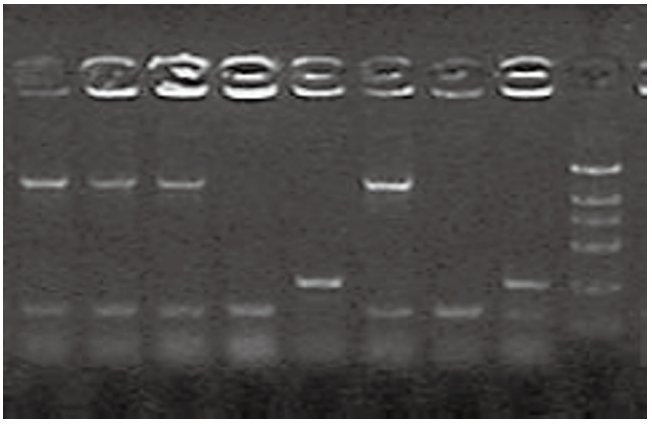

Figure 1. Colony of recombinant shuttle plasmid vector by PCR.

3.2. Enzyme identification of recombinant shuttle plasmid vector

It was digested by *Hin*d Ⅲ and *Xho* Ⅰ, and then identified

by agarose gel electrophoresis and sequencing. Sequencing results showed that recombinant shuttle plasmid carrier was successfully established (Figure 2).

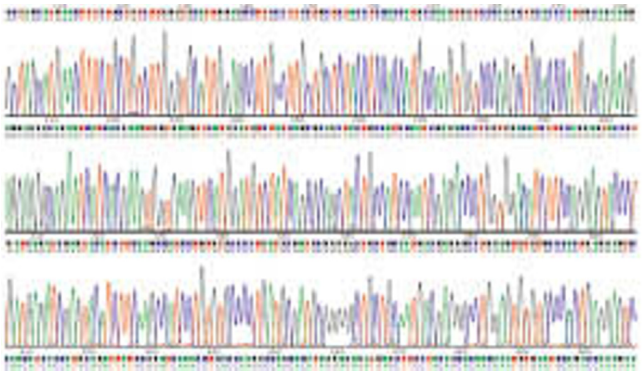

Figure 2. Recombinant shuttle plasmid vector sequence map.

3.3. Identification of recombinant plasmid pAdEasy-hVEGF165

The product of agarose gel electrophoresis was digested with *Pac* Ⅰ, and two fragments were obtained (Figure 3). It showed that recombinant plasmid was successfully established.

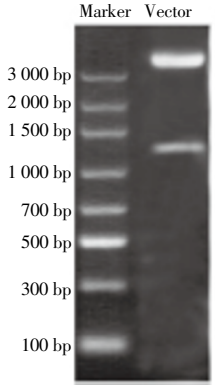

Figure 3. Detection of recombinant plasmid.

3.4. MRNA expression in HEK 293T cells

After transfection, total RNA of HEK 293T cells was detected by RT-PCR detection. It was found that VEGF165 had high expression, but it was not expressed in empty transfection group (Figure 4).

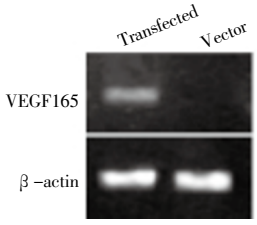

Figure 4. Expression of VEGF165.

3.5. Expression of GFP in the HEK 293T cell

24 h after transfection, cells showed green fluorescence expression (Figure 5a), and 6 days after transfection, all cells were positively expressed (Figure 5b).

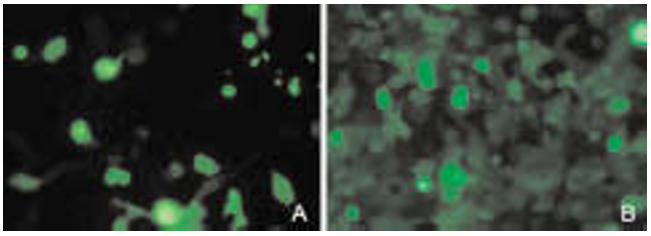

Figure 5. Expression of green fluorescence after transfection (×100). A: after 24 h; B: after 6 days.

3.6. Identification of bone marrow mesenchymal cells

Immunohistochemicalsaasy showed that SH3 had positive expression (Figure 6a). After mineralization and alizarin red staining, BMSCs positive staining was observed (Figure 6b).

Figure 6. BMSCs identification.
A: SH3 immunohistochemical staining (×400); B: Alizarin red staining (×200).

3.7. hVEGF gene expression after transfection

After transfection, BMSCs group showed VEGF expression, and no expression in control group (Figure 7).

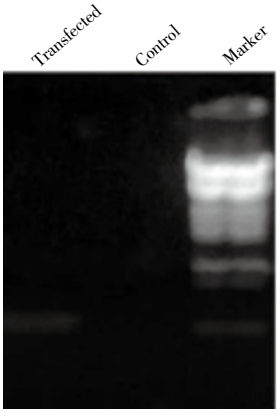

Figure 7. hVEGF gene expression after transfection.

3.8. BMSC VEGF protein expression after transfection

After transfection, positive staining cells showed brown cytoplasm and nucleus, and transfection of the control group showed no positive staining (Figure 8).

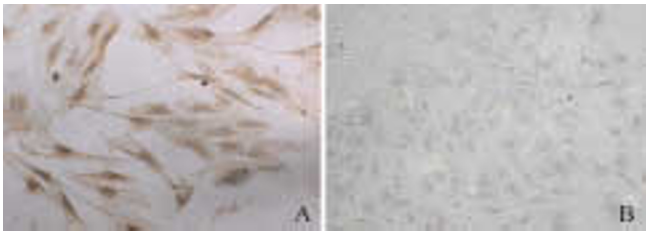

Figure 8. BMSC VEGF protein expression after transfection.
A: transfection group; B: not transfection group.

4. Discussion

In gene therapy study, efficiency and feasibility of gene transfection should be observed and evaluated. Gene report is common detection method. Common genes in report such as beta x-gal enzyme gene (LacZ) and luciferase gene (Lux) gene, should interact with specific substrates to produce detectable signals. GFP expression system is able to detect transfection efficiency and fluorescence intensity at the same time. It is rapid, convenient and of great repeatability, and can be used in quantitative analysis. It is ideal mark for detecting gene and protein expression and localization of in cells and tissues, and has been widely used in gene therapy, drug screening, and researches of transgenic animals, *etc*[3].

VEGF can carry signals by combining with specific receptors on the surface of the endothelial cells[4], so as to maintain normal blood vessels and integrity, increase vascular permeability, promote endothelial proliferation and angiogenesis. In recent years, studies have shown that VEGF protein injection or VEGF gene treatment in tissue repair and angiogenesis shows a good application prospect[5-7].

Ectomesenchymal stem cells are derived from adult bone marrow pluripotent stem cells in the bone marrow, because of its strong ability of differentiation and self-renewal ability and are widely used in theoretical research. Bone marrow mesenchymal stem cells have great potential in genetically modified in the treatment. It can modify MSCs, make its over-expression to induce apoptosis ligand and cytokines, *etc*. then it can effectively treat glioma, colon and prostate cancer[8-11]. It has been reported in treatment of brain ischemia and neurodegenerative diseases[12-14].

In conclusion, this study successfully constructed recombinant adenovirus vector of VEGF expressed in GFP and human, transformed VEGF into bone marrow mesenchymal stem cells with green fluorescent protein markers, and successfully constructed the VEGF-GFP-BMSCs. It helps real-time tracking after VEGF modified BMSCs in the repair tissue damage, its role in the treatment of related diseases. It has potential application value and clinical significance.

Conflict of interest statement

We declare that we have no conflict of interest.

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