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**Research Paper**

# Construction and identification of recombinant adenovirus-mediated gene transfer system for rat vascular endothelial growth factor☆

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## Abstract

Objective: To construct the recombinant adenovirus vector carrying rat vascular endothelial growth factor(VEGF), as preparation for genetic transfection that follows. Methods: Rat VEGF was obtained by using RT-PCR amplification and then cloned into the shutter plasmid pDC316. Subsequently, this newly constructed plasmid pDC316-VEGF, after identification by nuclease digestion analysis and sequencing analysis, was transfected into human embryonic kidney cells HEK293 by Lipofectamine 2000 mediation, together with adenovirus-packaging plasmid pBHGE3. Based on the homologous recombination of the two plasmids within HEK293 cells, the recombinant adenovirus vector carrying VEGF and VDC316-VEGF was created. VDC316-VEGF was subsequently identified using PCR, purified using repeated plaque passages, proliferated using freezing and melting within HEK293 cells, and titrated using 50% Tissue Culture Infective Dose(TCID50) assay. Results:The newly constructed recombinant adenovirus was confirmed to carry rat VEGF based on PCR results, and its titration value determined based on TCID50 assay was $3 \times 10^9$ pfu/ml. Conclusion:The recombinant adenovirus carrying rat VEGF was successfully constructed. The newly constructed adenovirus can produce a sufficiently high titration value within HEK293 cells, providing a reliable tool for genetic transfection in further gene therapy researches.

**Key words**: vascular endothelial growth factor; adenovirus vector; genetic transfection

## INTRODUCTION

Vascular endothelial growth factor(VEGF; a glucoprotein) was first separated and purified from media conditioned by bovine pituitary folliculostellate cells(FC) by Ferrara et al in 1989[1]. Researches over the years have confirmed that VEGF is one of those most critical secretary growth factors for the promotion of angiogenesis and vasculogenesis. It can with high specificity, promote vascular endothelial cell division, multiplication and migration, modulate extracellular matrix, and increase microvascular permeability as well. Thus VEGF has been the first choice in using gene therapy technologies to treat diseases, such as coronary heart disease, peripheral arterial occlusive disease (PAOD), restenosis(RS), limb replantation, and bone defects[2-6]. Endothelial progenitor cells(EPCs) are precusors of mature vascular endothelial cells and can function as a stem cell population with potential for both self-renewal and directed differentiation into mature endothelial cells[7-8]. Many researches in recent years have demonstrated that VEGF participated as a most important regulator to motivate EPCs to be released from bone marrow into the periferal blood and to induce differentiation of EPCs into matured endothelial cells[9-11]. Hideki et al[12] first attempted to use VEGF-transfected EPCs in a mouse model to treat experimently induced limb ischemia. They reported that VEGF-modified EPCs in vitro showed greatly improved ability for proliferation, motibilty and adehesion, and in vivo showed greatly enhanced capibility to repair injured vascular endothelial cells. In order to further explore

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the possibilty for the combined use of VEGF gene therapy and EPC stem cell therapy to speed up the process of repairing the damaged vascular endothelial cells, we have successfully developed the animal model of restenosis, and established the technology of separation, identification, and culture of EPC cells from periferal blood. To meet the required step of transfecting VEGF into EPC in following studies, it is intended in this study to construct a recombinant adenovirus vector carrying VEGF by co-transfecting two plasmids into human embryonic kidney cells HEK293.

## MATERIALS AND METHODS

### Plasmids, strains, and cells

Shutter plasmid pDC316, adenovirus-packaging plasmid pBHGE3, and human embryonic kidney cells HEK293 were obtained from Microbix Biosystems Co, Canada. The strain *E.coli* DH5α was kept in our lab.

### Enzymes and reagents Restriction enzymes

EcoR Ⅰ and Hind Ⅲ were from NEB Co, USA. PCR reagents Taq Polymerase and dNTPs were from Shanghai Biocolor BioScience & Technology Co, China. DNA Gel Recovery kit was from MN Co, Germany. The DNA ligase Solution I were from TaKaRa Co, Japan. Plasmid DNA Extraction Kit and Virus DNA Extraction Kit were from Qiagen Co, Germany. Lipofectamine 2000 Kit was from GIBCO BRLQIAGEN Co, USA.

### Amplification of VEGF from rat cDNAs by using RT-PCR

RT-PCR primers were designed to cover rat VEGF. The forward primer sequence was 5′-GGG AAT TCA TGA ACT TTC TGC TCT CTT GGG TGC-3′ and the reverse primer sequence was 5′-GGA AGC TTT CAC CGC CTT GGC TTG TCA CA-3′. Rat cDNAs was employed as templates for PCR amplification. PCR reaction was performed with 5 min of initial denaturation at 95℃, 35 cycles of 45 s denaturation at 94℃, 45 s annealing at 60℃, 45 s extension at 68℃, and finally 10 min extension at 68℃. RT-PCR amplification products were electrophoresed and inspected on a 1.1% agarose gel, and recovered and purified by using DNA Gel recovery kit.

### Construction and identification of plasmid containing VEGF gene, pDC316-VEGF

The recovered and purified PCR products and the shutter plasmid pDC316 were digested, respectively, with nucleases EcoRI and Hind Ⅲ in the 37℃ water bath for 8 h. The resulting two nuclease-digested products were then inspected and separated on a 1.1% agarose gel, and recovered by using DNA Gel Recovery Kit. Subsequently, the two recovered nuclease-digested products were incubated at 16℃ for 16 h after addition of Solution I containing DNA Ligase and the resulting ligated products were used to transfect well-prepared competent *E.coli DH5α*. The whole transfection mix was plated onto a prewarmed LB-ampicillin(AMP) agar plate and then incubated at 37℃ for 12 h. Individual growing colonies were picked out and incubated at 37℃ for 12 h in LB broth containing AMP. Finally, full-length plasmid DNA was extracted from positive clones using plasmid DNA extraction kit and then subject to testing of the presence of VEGF with both nuclease digestion using EcoRⅠ and Hind Ⅲ and DNA sequencing. The recombinant plasmid was designated as pDC316-VEGF after identification.

### Packaging, identification, proliferation, and titration of the recombinant adenovirus vector carrying rat VEGF, VDC316-VEGF

First, human embryonic kidney cells HEK293 were seeded into a 75 $cm^2$ culture flask containing DMEM supplemented with 10% FBS(10%FBS/DMEM), and cultured at 37℃ for 18-24 h in a $CO_2$ incubator(with $CO_2$ concentration adjusted at 5%) until cells grew to 60%~80% confluency. Then the HEK293 cells were transfected with the recombinant plasmid pDC316-VEGF by Lipofectamine 2 000 mediation, together with the adenovirus-packaging plasmid pBHGE3 containing the right arm of adenovirus type 5. After successful co-transfection, the HEK293 cells continued to be incubated for a period until the formation of viral plaques based on cytopathic effects(CPE) was visible. Big plaques were picked to perform three rounds of passages for virus purification before viral DNA was extracted using QIAamp DNA Blood Mini Kit. The extracted viral DNA was subsequently used for PCR identification. The primers and reaction conditions for PCR verification of VEGF were identical to those described above(see Amplification of VEGF from rat cDNAs by using RT-PCR). The newly constructed recombinant adenovirus was designated as VDC316-VEGF after identification. The process of repeated freezing and melting was adopted for proliferation of adenovirus in HEK293 cells to obtain desired virus amount. First, 0.5 ul virus stock solution was picked and diluted to 1ml with 10%FBS/DMEM, and then mixed well for preparation of virus mixture. Next, HEK293 cells were cultured in 10 ml 10%FBS/DMEM in a standard way. When the cell number reached $5 \times 10^6$, culture solution was discarded and the remained cells were mixed carefully with the prepared virus mixture by vortexing slowly three times. After the mixture was incubated at 37℃ for 120 min in a $CO_2$ incubator(with $CO_2$ concentration adjusted at 5%), 9 ml 10%FBS/DMEM was

added. The mixture continued to be incubated for additional 72 h before cells were collected and pelleted at 600 g for 5 min. Then the cells were resuspended in fresh preservative solution by performing three rounds of freezing and melting(-20℃ and 37℃). Finally, the cell debris was spinned down in a bench-top centrifuge at maximum speed for 20 min and the supernatant was collected. The whole procedure can be repeated many times until the desired virus amount is obtained. 50% tissue culture infective dose(TCID50) assay was emplyed to titrate the adenovirus. Firstly, a flask of HEK293 cells was collected, counted and diluted. Then 100 μl of cell suspension(about $1\times10^4$ in cell amount) was transferred into each well of a 96-well plate and the plate was incubated at 37℃ in an incubator, ready for use. Next, a 10-fold dilution series was made for virus stock until dilution fold reached $10^{-10}$. The last 8 dilution samples were transferred to the prepared 96-well plate with HEK293 cells. For each dilution, 0.1 ml of the diluted virus was added to each of the 10 wells of the row plus 2 negative controls. The presense of CPE was observed and counted under an inverted microscope after the 96-well plate was incubated in an incubator at 37℃ for 10 days. Finally, the titration value for the virus was calculated according to Karbers' Equation.

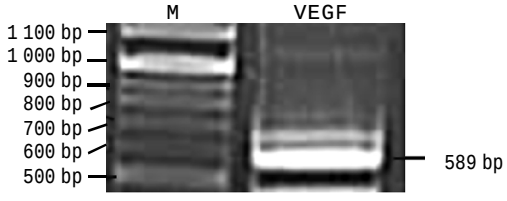
## RESULTS

### Amplification of VEGF from rat cDNAs by using RT-PCR

Based on the published reference sequence of rat VEGF in GeneBank and the locations of the forward and reverse primers for PCR amplification, the predicted size of PCR product should be 589 bp. It was shown from agarose gel electrophoresis result of PCR products (Fig 1) that there existed an electrophoretic band around 600 bp(indicated by an arrow), confirming the successful PCR amplification of rat VEGF.

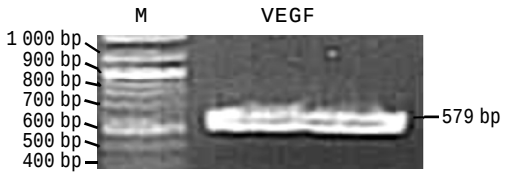
### Construction and identification of the plasmid containing VEGF gene, pDC316-VEGF

The recovered and purified PCR products after using DNA Gel Recovery Kit and the shutter plasmid pDC316 were supposed to yield two fragments of 579 bp and 3 895 bp, respectively, after the double nuclease digestion with *EcoR* Ⅰ and *Hind* Ⅲ. It was shown from agarose gel electrophoresis result(Fig 2, Fig 3) that the sizes of the fragments after nuclease digestion were consistent with predictions. Next, competent *E.coli DH5α* was tranfected by the ligated two nuclease digestion products and the resulting positive *E.coli* clones after culturing for 12 h should contain the recombinant plasmid carrying VEGF, i.e. PDC316-VEGF. PDC316-VEGF was predicted to yield one fragment of 4 474 bp after nuclease digestion of EcoRI, and yield two bands of 579 bp and 3 895 bp, respectively, after double nuclease digestion of *EcoR*I and *Hind* Ⅲ. This prediction was verified by agarose gel electrophoresis result(Fig 4). Further verification was performed by DNA sequencing and the sequencing result substantiated that PDC316-VEGF contained a DNA fragment totally identical in sequence to that of rat VEGF published in Genebank.



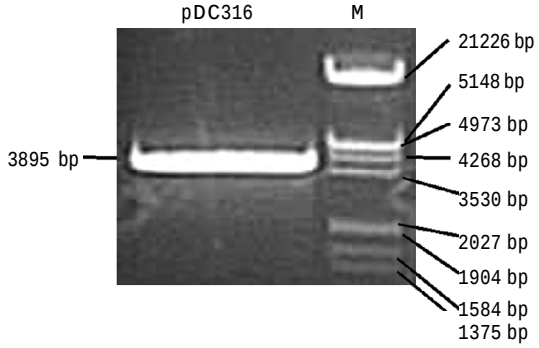

M:100 bp DNA Marker(NEB); VEGF: Electrophoresis result of RT-PCR product of VEGF gene.

Fig 1 Electrophoresis result of RT-PCR amplification of VEGF gene




M:100 bp DNA Marker(NEB); VEGF: Electrophoresis result of RT-PCR product on VEGF after double nuclease digestion.

Fig 2 Electrophoresis result of RT-PCR product of VEGF gene after double nuclease digestion of *EcoR* Ⅰ and *Hind* Ⅲ

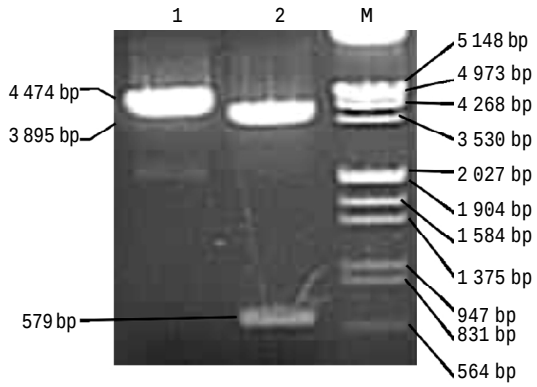



M: λ DNA/EcoR Ⅰ +*Hind* Ⅲ(MBI); pDC316: Electrophoresis result of pDC316 after double nuclease digestion.

Fig 3 Electrophoresis result of pDC316 after double nuclease digestion of *EcoR* Ⅰ and *Hind* Ⅲ

### Packaging, identification, proliferation, and titration of the recombinant adenovirus vector carrying rat VEGF, VDC316-VEGF

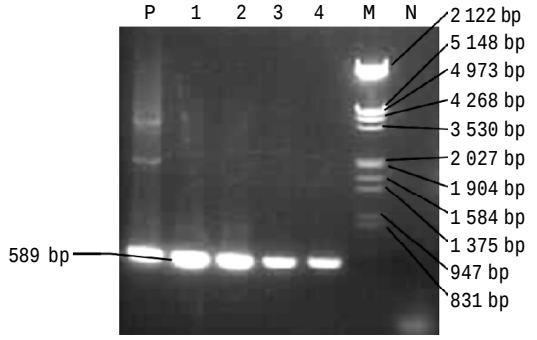
Virus plaques based on CPU were observed 9-14 days after newly constructed shutter plasmid pDC316-cDNA165 was transfected into human embryonic kidney cells HEK293 by Lipofectamine 2 000 mediation,




M: λNA/*EcoR* Ⅰ+*Hind* Ⅲ(MBI); 1:Electrophoresis result of PDC316-VEGF after digestion of *EcoR* Ⅰ; 2: Electrophoresis result of PDC316-VEGF after digestion of *EcoR* Ⅰ and *Hind* Ⅲ.

Fig 4 Electrophoresis result of PDC316-VEGF after nuclease digestion of *EcoR* Ⅰ only, and both *EcoR* Ⅰ and *Hind* Ⅲ

together with adenovirus-packaging plasmid pBHGE3. After three passages, virus plaques were subsequently subject to PCR verification of the existence of VEGF. Agarose gel electrophoresis results of PCR products(Fig 5) showed that all of the four samples could yield a PCR fragment identical to that of the positive control, indicating that the recombinant adenovirus was successfully constructed. After its proliferation using repeated freezing and melting, the adenovirus was subject to titraton using TCID50 assay. The titrated value was 3 × 10^9pfu/ml, which was high enough for gene transfer requirement in later experiments.




P:product on VEGF from PDC316-VEGF; 1-4: Samples 1-4; M: λNA/*EcoR* Ⅰ+*Hind* Ⅲ(MBI); N: negative control.

Fig 5 Electrophoresis result of PCR verification of VDC316-VEGF.

## DISCUSSION

VEGF, as a mitogenic factor in high specificity for vascular endothelial cell, has a strong confirmed ability to promote the growth of vascular endothelial cells and induce angiogenesis and vasculogenesis both in vitro and in vivo. For the time being six different sorts of transcripts have been identified for human VEGF: VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, VEGF206[13]. Among these transcripts, VEGF165 is most abundantly expressed in majority of cell types, and has the strongest capability for promoting division of endothelial cells[14].

In order to further explore the possibilty for the combined use of VEGF gene therapy and EPC stem cell therapy to speed up the process of repairing the damaged vascular endothelial cells, we have successfully developed the animal(rat) model of restenosis, and established the technology of separation, identification, and culture of EPC cells from rat periferal blood. Thus, rat VEGF was chosen as the target gene for constructing recombinant adenovirus in this experiment to meet the required step of transfecting VEGF into EPC in following studies.

In recent years the application of gene therapy has opened up a brand-new area for treamtment of vascular diseases. One of those key factors for carrying out gene therapies successfully is the vector chosen for gene transfer. At present virus vector, including retrovirus, adenovirus, and adeno-associated viruses(AAV), has been the common choice in gene therapies in clinical experiments and trials. Adenovirus is specifically the most widely used virus vector based on its following advantages[15-18]: ① is relatively easy to construct, and can be growed at high titers;② has a wide host range, and can efficiently infect both quiescent and actively dividing cells; ③ has a genome of big size, and therefore can accommodate reasonably large foreign inserts (up to 8 kb);④ allows high expression of the recombinant protein in host cells; ⑤remains epichromosomal, i.e. does not integrate into the host chromosome, thus reducing the risk of insertional mutagenesis. However, there is no denying that adenovirus vector is afflicted with many disadvantages. One of the major disadvantages are the viral proteins(E1,E2 and E4) encoded by the virus genome itself. Viral proteins, as foreign antigens, can result in variable degrees of immugenic reactions in host organisms. Therefore, the construction of new adenovirus vectors has always been of high interest and emphasis in the gene therapy research field. In the newest generation of adenovirus vector available to today's use, all of the coding sequence within the virus genome is deleted and only the repeat sequence ITR and the virus packaging sequence are intact, thus the immugenity of the vector can be decreased to the lowest possible level[19]. We can expect that the next generation of adenovirus vector with higher efficiency and safety could be available for gene therapy in the near future.

In this experiment the construction of adenovirus was based on the homologous recombination between two plasmids in E1-complementing cell lines[20]. The basic

procedure is as follows: firstly, a shutter plasmid pDC316 with deleted E1 region that carries VEGF was transfected into the E1-complementing cell line HEK293 by Lipofectamine 2000 mediation, together with an adenovirus packaging plasmid pBHGE3 containing the right arm of adenovirus type 5; next, the recombinant adenovirus genome was generated from the homologous recombination between the two plasmids and packaged into the viral article within HEK293 cells. In this experiment two measures were taken to simplify the manipulation procedures and increase the recombination efficiency. The first measure was the introduction of the recognition sequences for nucleases *EcoR* Ⅰ and *Hind* Ⅲ in the forward and reverse PCR primers, respectively, which facilited the ligation of VEGF into the shutter plasmid pDC316. The second measure was the use of the cationic lipid Lipofectamine 2000 in the co-transfection of two plasmids into HEK293, which greatly increased the upptake of null DNA by mammalian cells.

In this experiment the recombinant adenovirus vector VDC316-VEGF was successfully constructed, and it could grow at relatively high titers in HEK293 cells. The newly constructed vector provides the prerequisite for gene transfer requirement in future studies.

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