Construction and identification of pIRES$_2$-GDNF-VEGF$_{165}$ bicistronic eukaryotic expression vector

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Abstract

BACKGROUND: Human glial cell line-derived neurotrophic factor (GDNF) and vascular endothelial growth factor 165 (VEGF$_{165}$) are essential genes for cell differentiation.

OBJECTIVE: To construct and identify pIRES$_2$-GDNF-VEGF$_{165}$ bicistronic eukaryotic expression vector.

METHODS: Human GDNF genes were obtained from the genomic DNA of human peripheral blood mononuclear cells by PCR. Then the GDNF cDNA fragment was inserted into the multiple cloning sites of pIRES$_2$-EGFP, to generate the bicistronic eukaryotic expression plasmid pIRES$_2$-GDNF-EGFP. The VEGF$_{165}$ gene was obtained from pIRES$_2$-VEGF$_{165}$-EGFP plasmid by twin PCR. Then VEGF$_{165}$ cDNA fragment was cloned into the pIRES$_2$-GDNF-EGFP, instead of EGFP, to create a double gene co-expressing vector plasmid pIRES$_2$-GDNF-VEGF$_{165}$ containing internal ribosome entry sites. Then pIRES$_2$-GDNF-VEGF$_{165}$ was used to transfect HEK293 cells. RT-PCR and western blot analysis were performed to test the co-expression of double genes.

RESULTS AND CONCLUSION: DNA sequencing analysis demonstrated that the GDNF and VEGF$_{165}$ were exactly consistent with the sequence recorded in the GenBank. The size of GDNF gene was 636 bp and the size of VEGF$_{165}$ gene was 576 bp. Enzyme digestion analysis indicated that, pIRES$_2$-GDNF-VEGF$_{165}$ bicistronic eukaryotic expression vector inserted GDNF band by *Bgl* II/*Bam* HI, inserted IRES-VEGF$_{165}$ fragment by *Bam* HI/*Not* I, and inserted GDNF-IRES-VEGF$_{165}$ fragment by *Bgl* II/*Not* I. RT-PCR and western blot analysis showed that, after HEK293 cells were transfected with pIRES$_2$-GDNF-VEGF$_{165}$, double genes were expressed at the mRNA and protein levels. The pIRES$_2$-GDNF-VEGF$_{165}$ bicistronic eukaryotic expression vector is successfully constructed.

Subject headings: glial cell line-derived neurotrophic factor; vascular endothelial growth factor; carrier proteins; tissue engineering

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INTRODUCTION

Spinal cord injury can cause severe spinal nerve dysfunction[1]. With the development of molecular therapy, gene therapy is considered to be a new choice for treatment of spinal cord injury[2]. Vascular endothelial growth factor (VEGF) has the effect of promoting angiogenesis[3]. Increasing evidence of recent studies showed that, VEGF also can promote the growth of nerve cells[4]. On the one hand, VEGF can stimulate the regeneration nerve and its nutrient vessels to repair spinal cord injury[5]; on the other hand, VEGF can increase the activity of peripheral nervous system nerve cells through vegetative nerve and mitogen activation[6].

Glial cell line derived neurotrophic factor (GDNF) is a novel neurotrophic factor, belongs to TGF-p superfamily, which was isolated and purified from the supernatant from rat B49 cells in 1993 by Lin *et al*[7-10]. GDNF is one of the most effective neurotrophic factors for supporting the growth of motor neurons *in vitro*, it can not only save the programmed death of motor neurons during the development process, but also promote the survival of cortical neurons after spinal cord injury[11-15]. In addition to nutritional and protective effect on the midbrain dopaminergic neurons, GDNF also has nutritional and protective effect to different degree on the sensory and motor neurons[16-18].

In this study, we constructed the co-expression plasmid of GDNF and VEGF, and then transfected HEK293 cells and detect the expression. This provides the experimental

basis for the following experiment of co-transfecting mesenchymal stem cells, improving the internal environment of spinal cord injury, regulating the differentiation of mesenchymal stem cells, increasing the number of newborn neurons, and promoting functional recovery of spinal cord.

MATERIALS AND METHODS

Design

A gene experiment.

Time and setting

The experiment was completed in the Life Science College of Xinxiang Medical University, China from November 2011 to August 2013.

Materials

Escherichia coli DH5α are kept in Stem Cells and Biological Treatment Center at Xinxiang Medical University, China. Fresh anticoagulant blood samples are donated from a volunteer in this project group. This volunteer is a 30-year-old male, with no disease previously. There was no disease detected by physical examination. We have informed the donor of the purpose, significance and possible discomfort of this experiment, and have got the permission of this volunteer, who signed the informed consent.

Vector

pIRES$_2$-EGFP plasmid was purchased from Beijing TianDz Inc. pIRES$_2$-VEGF$_{165}$-EGFP plasmid are preserved in Stem Cells and Biological Treatment Center at Xinxiang Medical University in China.

Cells

HEK293 cells are kept in Stem Cells and Biological Treatment Center at Xinxiang Medical University in China.

Related reagents and instruments on construction experiment of eukaryotic expression vector on double gene consist of GDNF and VEGF$_{165}$:

Reagents and instruments	Source
T4 DNA ligase	Takara Biotechnology (Dalian) Co., Ltd.
Prime STAR Max DNA polymerase, *Bam* HI, *Bst* XI, *Bgl* II, *Not* I, Total RNA extraction kit, High-purity gel extraction kit, DNA marker, Lipofectamine™ 2000	Invitrogen
Nano Drop2000 UV-Vis Spectrophotometers	Thermo Fisher Scientific
NBS	Hangzhou Sijiqing Biological Products

Methods

Design of the primers

GDNF (NM_000514.3) and VEGF$_{165}$ gene sequence (NM-001025368.2) in Gene Bank served as the template and was used to design the primers. Overlap-PCR, an efficient and rapid method, was used to clone human GDNF gene CDS (coding sequence) from genomic DNA. The procedure included four primers and three-step PCRs. The Coding Sequence of human GDNF gene fragment includes two exons (exon1 + exon2). Two primer pairs (forward-primer and reverse-primer) were designed to amplify GDNF-1 (exon1), *Bgl* II restriction enzyme cut site was added in forward-primer which is the outermost primers in the upstream. Other two primer pairs (forward-primer and reverse-primer) were designed to amplify the exon2 (GDNF-2). SmaII restriction enzyme cut site was added in reverse-primer which is the outermost primers in the downstream. The length of amplified fragment was 636 bp.

Two primer pairs were designed to amplify the human VEGF$_{165}$ from the pIRES$_2$-VEGF$_{165}$-EGFP. Forward-long primer was 4 bases longer than forward-short at the 5' end, so did primer reverse-long than reverse-short. The forward primers were introduced parts of sequence of *Bst* XI site, and in reverse primers of *Not* I site, which will be used to assemble the *Bst* XI and *Not* I sticky ends. The length of amplified fragment was 576 bp.

Primers of GDNF and VEGF$_{165}$:

Gene	Primer sequence (5'–3')
GDNF-1 (exon1)	Forward: GAA GAT CTA TGA AGT TAT GGG ATG TCG TG Reverse: TCT GGC ATA TTT GAG TCA CTG CTC AG
GDNF-2 (exon2)	Forward: GAG CAG TGA CTC AAA TAT GCC AGA Reverse: TTC CCG GGT CAG ATA CAT CCA CAC CTT T
VEGF$_{165}$ (1)	Forward: ATG AAC TTT CTG CTG TCT TGG GTG C Reverse: GCT CAC CGC CTC GGC TTG TCA
VEGF$_{165}$ (2)	Forward: AAC CAT GAA CTT TCT GCT GTC TTG GGT GCA TT Reverse: GGC CGC TCA CCG CCT CGG CTT G

Construction of pIRES$_2$-GDNF-EGFP

The genomic DNA of human peripheral blood mononuclear cells severed as the template and the primer of GDNF was used to amplify GDNF gene. In this study, Overlap-PCR, an efficient and rapid method, was used to clone human GDNF gene CDS (coding sequence) from genomic DNA. The procedure included four primers and three-step PCRs. Human GDNF gene consists of two exons and the CDS contains 636 bp. In the first step three PCRs were performed to generate extended exon1 (GDNF-1), exon2 (GDNF-2) that contained overlapped nucleotides and were used as the templates for second ligation PCR. Two primer pairs were designed to amplify exon1 (GDNF-1). Other two primer pairs were designed to amplify the exon2 (GDNF-2). Secondly, exon1 (GDNF-1) and exon2 (GDNF-2) were spliced together. Lastly, the two exons (GDNF-1 and GDNF-2) were linked together with outermost primers and the templates from the second step. As an efficient and rapid method, overlap-PCR is feasible and acceptable for gene cloning from genomic DNA.

The genomic DNA of human peripheral blood mononuclear cells severed as the template and the primer of GDNF was used to amplify GDNF gene. A total of 20 μL PCR reaction system was added, including genomic DNA 0.5 μL (10 ng), 2 × Prime STAR Max DNA Polymerase 10 μL, RNase-Free Water 7.5 μL, upstream primer and downstream primer 2 μL. The reaction conditions were: 95℃ for 5 minutes, 98 ℃ for 10 seconds, 55 ℃ for 5 seconds, and 72 ℃ for 55 seconds, 30 cycles; finally, maintained at 72 ℃ for 5 minutes. PCR product was purified by the PCR Purification Kit, then the PCR product and plasmid pIRES$_2$-EGFP was cut by *Bgl* II and *Sma* I. After digestion, PCR product was purified by the PCR Purification Kit. The DNA fragment was inserted into the plasmid by T4 DNA ligase, 20 μL system was added, including pIRES$_2$-EGFP 2 μL, cDNA (GDNF) 8 μL, T4 DNA ligase 0.2 μL, at 22 ℃ for 30 minutes. The DNA fragment was translated into *Escherichia coli* DH5α and placed on LB plate (kanr) at 37 ℃ incubator for 16 hours. Monoclonal colony was picked up and shaken for 12−16 hours at 37 ℃ with a speed of 225 r/minute. The plasmid was extracted and identified by using *Bgl* II and *Bam* HI double enzyme digestion. The recombinant plasmid pIRES$_2$-GDNF-EGFP was obtained.

PCR amplification of VEGF$_{165}$

Two parallel PCRs were set up using forward-long/ forward-short or reverse-long/reverse-short primer pairs, with the pIRES$_2$-VEGF$_{165}$-EGFP plasmid by as templates. Both amplifications were subjected to 30 cycles of 94 ℃ for 30 seconds, 54 ℃ for 30 seconds and 72 ℃ for 1 minute, followed by a 5 minute extension at 72 ℃ using Prime STAR Max DNA Polymerase. Another two parallel PCRs were performed using EXTaq DNA polymerase. The expected DNA fragments in the four PCR products were purified separately using DNA gel extraction kit and quantified by Nano Drop 2000 UV-Vis Spectrophotometers.

Construction of pIRES$_2$-GDNF/VEGF$_{165}$

PIRES$_2$-GDNF-EGFP was digested with *Bst* XI and *Not* I, followed by phenol: chloroform extraction and ethanol precipitation. Meanwhile, each pooled VEGF1$_{65}$ PCR products amplified with Prime STAR Max DNA Polymerase were mixed, denatured at 94 ℃ for 4 minutes and re-annealed at 65 ℃ for 2 minutes. For a ligation reaction, the annealed VEGF$_{165}$ and linearized pIRES$_2$-GDNF-EGFP (molar ratio equal to 4:1) were incubated with T4 DNA ligase at 16 ℃ for 3 hours. The two ligation products were used to transform *Escherichia coli* DH5a $CaCl_2$ competent cells following standard method. Screening of the transformants was performed by *Bst* XI plus *Not* I digestion following alkalinelysis plasmid preparation, and the recombinants were confirmed by DNA sequencing.

Two parallel PCRs were set up using either forward-long/reverse-long (PCR products A) or forward-short/reverse-short primer pairs (PCR products B), with the pIRES$_2$-VEGF$_{165}$-EGFP plasmid (double ring means this plasmid in this figure) by as templates. Equimolar of the two PCR products A and B were mixed, heat denatured and re-annealed to generate hybridized DNA fragments. Four types of double-stranded DNA molecules with equal proportions are generated *via* random complementary pairing. They are molecule I, II, III and IV. Two of them, I and II, are blunt-ended and another two are sticky-ended. The molecule III possess a 5' *Bst* XI sticky end and a 3' *Not* I sticky end, which allow the fragment to be cloned directionally into pIRES$_2$-BDNF-EGFP digested with *Bst* XI and *Not* I.

In vitro transduction of HEK293 cells

HEK293 cells were maintained in Dulbecco's modified Essential Medium (DMEM) containing 10% newborn bovine serum and 100 mg/L penicillin/streptomycin. Cells were maintained in a humidified environment at 37 ℃ and 5% CO_2. Cell viability was monitored with trypan blue exclusion method. The viability was over 95% in all experiments. Cells were seeded at a density of 5 × 10^5 cells/well in a six-well tissue culture plate and cultured for 24 hours to 60%−80% confluence. HEK293 cells were either mock infected or infected with the pIRES$_2$-EGFP, pIRES$_2$-GDNF/EGFP and pIRES$_2$-GDNF/VEGF$_{165}$ three vectors using Lipofectamine^{TM} 2000 respectively for 2 hours at 37 ℃ at 5 μg per well. Two hours later, the transfection medium was removed, and fresh complete growth medium was added. Twenty-four hours post-transfection, they were observed under the inverted fluorescent microscope. The expression of the BDNF and NT-3 gene was detected by RT-PCR and western blot analysis 3 days later.

RT-PCR detection of the expression of GDNF and VEGF$_{165}$ in HEK293 cells

To detect the GDNF and VEGF$_{165}$ mRNA expression levels in HEK293 cells which were either mock infected or infected with the pIRES$_2$-EGFP, pIRES$_2$-GDNF/EGFP and pIRES$_2$-GDNF/VEGF$_{165}$ respectively, the GDNF and VEGF$_{165}$ expression was primarily assessed by means of RT-PCR. The collected cells were added per 1 mL TRIZOL reagent and pipetting repeatedly, total cellular RNA was extracted in accordance with the instruction of manual steps. Then the extracted total RNA was transcribed and reversed into cDNA. Then the reverse transcription of the cDNA was amplified with the PCR, and the reaction conditions were pre-denaturation under 94 ℃ for 5 minutes; 94 ℃ for 30 seconds, 55 ℃ for 30 seconds, 72 ℃ for 30 seconds, 35 cycles, followed by 72 ℃ extension for 10 minutes. 5 μL of PCR amplification product was added with 1 μL loading buffer, placed in 20 g/L agarose gel electrophoresis at 100 V. Subsequently the cells were observed with gel imaging system, and the absorbance value of each band was analyzed.

Western blot analysis of the expression of GDNF and VEGF$_{165}$ in HEK293 cells

A standard western blot protocol was used to detect BDNF

and NT-3 protein expression in HEK293 cells which were either mock infected or infected with the pIRES$_2$-EGFP, pIRES$_2$-GDNF/EGFP and pIRES$_2$-GDNF/VEGF$_{165}$ vectors respectively. We collected the culture supernatants of infected HEK293 cells without serums for western blot analysis, which was assayed using an anti-GDNF and anti-NT-3 antibody. The 20 μg protein extracted from the culture supernatants of transduced HEK293 cells was separated on SDS-PAGE under reducing and denaturing conditions, and transferred to polyvinylidene difluo-ride (PVDF) membrane. The membranes were incubated in a 1:500 dilution of polyclonal rabbit anti-GDNF antibody (Santa Cruz Biotechnology, USA) or 1:250 dilution of polyclonal rabbit anti-VEGF$_{165}$ antibody (Santa Cruz Biotechnology), polyclonal rabbit anti-β-actin (1:500, Santa-Cruz Biotechnology) was used as the loading control. After washing with TBS for three times (10 minutes per time), cells were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (1:5 000) at room temperature for 2 hours. After TBS washing (3 times, for 15 minutes each), X-ray film was performed to observe the final results. The amount of signal was quantified with Gel-Pro analyzer software.

Main outcome measures

Expressions of GDNF and VEGF$_{165}$ mRNA and protein were detected by RT-PCR and western blot assay.

Statistical analysis

We using SPSS 13.0 statistical software on experimental data for statistical processing, the data are described in mean±SD, data comparisons between groups were tested by one-way analysis of variance. A $P < 0.05$ was considered statistically significant difference.

RESULTS

Amplification of GDNF and VEGF$_{165}$ genes

GDNF gene was obtained from the genomic DNA of human peripheral blood mononuclear cells by overlap PCR. The size of GDNF gene was 636 bp. The VEGF$_{165}$ gene was obtained from pIRES$_2$-VEGF$_{165}$-EGFP plasmid by twin PCR, and the size of VEGF$_{165}$ gene was 576 bp (**Figure 1**).

Identification of plasmid pIRES$_2$-GDNF-EGFP

The plasmid pIRES$_2$-GDNF-EGFP was cut by *Bgl* II and *Bam* HI double enzyme. A gene fragment with 636 bp was obtained, which was in full agreed with GDNF gene (**Figure 2A**).

Identification of plasmid pIRES$_2$-GDNF-VEGF$_{165}$

The plasmid pIRES$_2$-GDNF-VEGF$_{165}$ was cut by *Bgl* II and *Bam* HI double enzyme. And a fragment about 636 bp would be obtained. This indicated GDNF gene inserted into the plasmid pIRES$_2$-GDNF-VEGF$_{165}$. The sequence of the plasmid pIRES$_2$-GDNF-VEGF$_{165}$ was in accordance with gene sequence in Gene Bank. The plasmid pIRES$_2$-GDNF-VEGF$_{165}$ was cut by *Bam* HI and *Not* I double enzyme. And a fragment about 1 183 bp would be obtained. This indicated IRES-VEGF$_{165}$ gene inserted into the plasmid pIRES$_2$-GDNF-VEGF$_{165}$. The plasmid pIRES$_2$-GDNF-VEGF$_{165}$ was cut by *Bgl* II and *Not* I double enzyme. And a fragment about 1 825 bp would be obtained. This indicated BDNF-IRES-VEGF$_{165}$ gene inserted into the plasmid pIRES$_2$-GDNF-VEGF$_{165}$. The sequence of the plasmid pIRES$_2$-BDNF-VEGF$_{165}$ was in accordance with gene sequence in Gene Bank (**Figure 2B**).

RT-PCR analysis of the expression of GDNF and VEGF$_{165}$ in HEK293 cells

To illustrate mRNA expression by pIRES$_2$-EGFP, pIRES$_2$-GDNF/EGFP, and pIRES$_2$-GDNF/VEGF$_{165}$ transduced HEK293 cells, we evaluated the expression of GDNF and VEGF$_{165}$ by RT-PCR analysis. RT-PCR was performed using GDNF-specific primers and the β-actin sequence as an internal standard. GFP expression was monitored in pIRES$_2$-EGFP and pIRES$_2$-GDNF/EGFP transduced HEK293 cells by inverted fluorescence microscopy. Expression of GDNF mRNA was higher in either pIRES$_2$-GDNF/EGFP or pIRES$_2$-GDNF/VEGF$_{165}$ transduced HEK293 cells than that pIRES$_2$-EGFP transduced HEK293 cells or negative control (**Figure 3A**, **B**). As shown above, expression of VEGF$_{165}$ mRNA was higher in pIRES$_2$-GDNF/VEGF$_{165}$-transduced HEK293 cells than the other three (**Figure 3C**, **D**). These results demonstrated that the GDNF and VEGF$_{165}$ had been introduced successfully into HEK293 cells by pIRES$_2$-GDNF/EGFP and pIRES$_2$-GDNF/VEGF$_{165}$. After the HEK293 cells were transduced by pIRES$_2$-GDNF/EGFP, we passaged them continually and then monitored the mean percentage of expression of GFP under fluorescence microscopy. There was no decrease in GFP fluorescence, illustrating the maintenance of transgenic expression in the transduced cells.

Western blot analysis of the expression of GDNF and VEGF$_{165}$ in HEK293 cells

To illustrate protein expression by pIRES$_2$-EGFP, pIRES$_2$-GDNF/EGFP, and pIRES$_2$-GDNF/VEGF$_{165}$ transduced HEK293 cells, we evaluated the expression of GDNF and VEGF$_{165}$ by western blot assay. After 72 hours of transfection, we collected the culture supernatants of infected HEK293 cells without serums which were processed for western blot analysis using an anti-GDNF and anti-VEGF$_{165}$ antibody. β-actin served as an internal standard. The results suggested that exogenous GDNF protein was strongly expressed in pIRES$_2$-GDNF/EGFP and pIRES$_2$-GDNF/VEGF$_{165}$ transduced HEK293 cells, but the pIRES$_2$-EGFP transduced HEK293 cells and no transduced cells, the expression of endogenous GDNF was very low (**Figures 4A**, **B**). As shown above, western blot analysis also revealed that exogenous VEGF$_{165}$ protein was strongly expressed in pIRES$_2$-GDNF/VEGF$_{165}$ transduced HEK293 cells, and expression of endogenous VEGF$_{165}$ was very low in the pIRES$_2$-EGFP, pIRES$_2$-GDNF/EGFP transduced HEK293 cells (**Figures 4C**, **D**). These results also demonstrated that GDNF and VEGF$_{165}$ had been introduced successfully into HEK293 cells by pIRES$_2$-GDNF/VEGF$_{165}$.

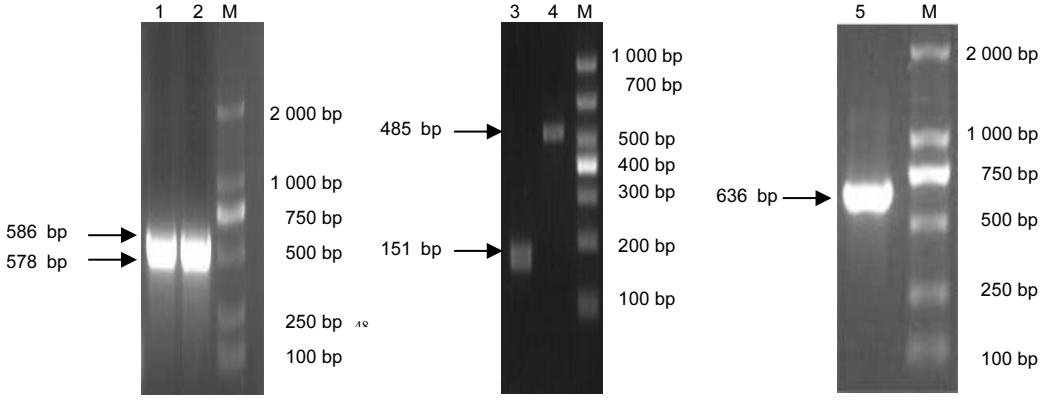

Figure 1 The amplification of GDNF and VEGF$_{165}$

Note: M: Marker; Number 1: VEGF$_{165}$ (forward-short/reverse-short) gene (578 bp); Number 2: VEGF$_{165}$ (forward-long/reverse-long primer pairs) gene (586 bp); Number 3: GDNF-1 (exon 1) gene (151 bp); Number 4: GDNF-2 (exon 2) gene (485 bp); Number 5: GDNF (exon1 + exon 2) gene (636 bp). GDNF: Glial cell line derived neurotrophic factor; VEGF: vascular endothelial growth factor.

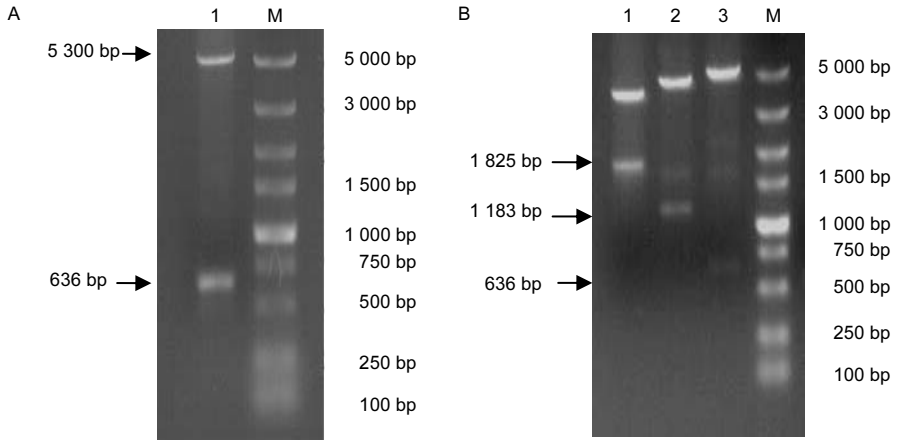

Figure 2 The identification of plasmid pIRES$_2$-GDNF-EGFP and pIRES$_2$-GDNF-VEGF$_{165}$

Note: (A) M: Marker; Number 1: pIRES$_2$-GDNF-EGFP cut by *Bgl* II and *Bam* HI double Enzyme. (B) M: Marker; Number 1: pIRES$_2$-GDNF-VEGF$_{165}$ cut by *Bgl* II and *Not* I; Number 2: pIRES$_2$-GDNF-VEGF$_{165}$ cut by *Bam* HI and *Not* I; Number 3: pIRES$_2$-GDNF-VEGF$_{165}$ cut by *Bgl* II and *Bam* HI. GDNF: Glial cell line derived neurotrophic factor; VEGF: vascular endothelial growth factor.

DISCUSSION

In this article, VEGF$_{165}$ was directly inserted into pIRES$_2$-GDNF-EGFP at the *Bst* XI and *Not* I sites. Those more efficient and economical enzymes, such as *Bst* XI and *Not* I, could always be chosen for vector digestion. It is not any longer necessary to buy so many kinds of restriction enzymes for cloning. In addition, interested inserts are amplified using proof-reading DNA polymerase, which provides a faithful guarantee for the PCR products. These advantages make it a universal technique for the directional cloning of PCR products.

GDNF is a protein found in recent years and has been cloned its gene, originally isolated from mouse glial cell line B49 cells, *in vitro* can activate the uptake of lateral dopamine neurons in ventral embryonic rats, and can promote neuronal survival[20]. This factor is composed of two monomers by glycosylation two sulfur bond into two body, monomer is composed of 134 amino acids[21]. The seven cysteine residues in the molecule conformation and transforming growth factor superfamily, and its sequence homology near 20%, belongs to the TGF superfamily distant from the structure[22]. The experimental study indicated that GDNF is a neurotrophic factor with multi effect, which function on the sensory neurons of nutrition, exercise and dopamine, and its effect is the change in the different period of individual development[23]. Because GDNF is a protein, not through the blood brain barrier and spinal cord barrier, also cannot be administered through the digestive tract, it must be through the effective methods to make it into the spinal cord or the surrounding, thus playing the best biological effect[24]. The current administration methods are: (1) direct or indirect perfusion: concrete ways is intraventricular, cerebellomedullary cistern and a margin of administration, because this method in the operation is complicated and can increase the infection, aggravate the injury of spinal cord, so its application is limited[25]. (2) The method of gene therapy for spinal cord injury is to cell transplantation of genetically modified GDNF such as glial cells, fibroblasts, thus providing micro-environment to promote neuronal growth, the method is obviously better than that of perfusion method[26].

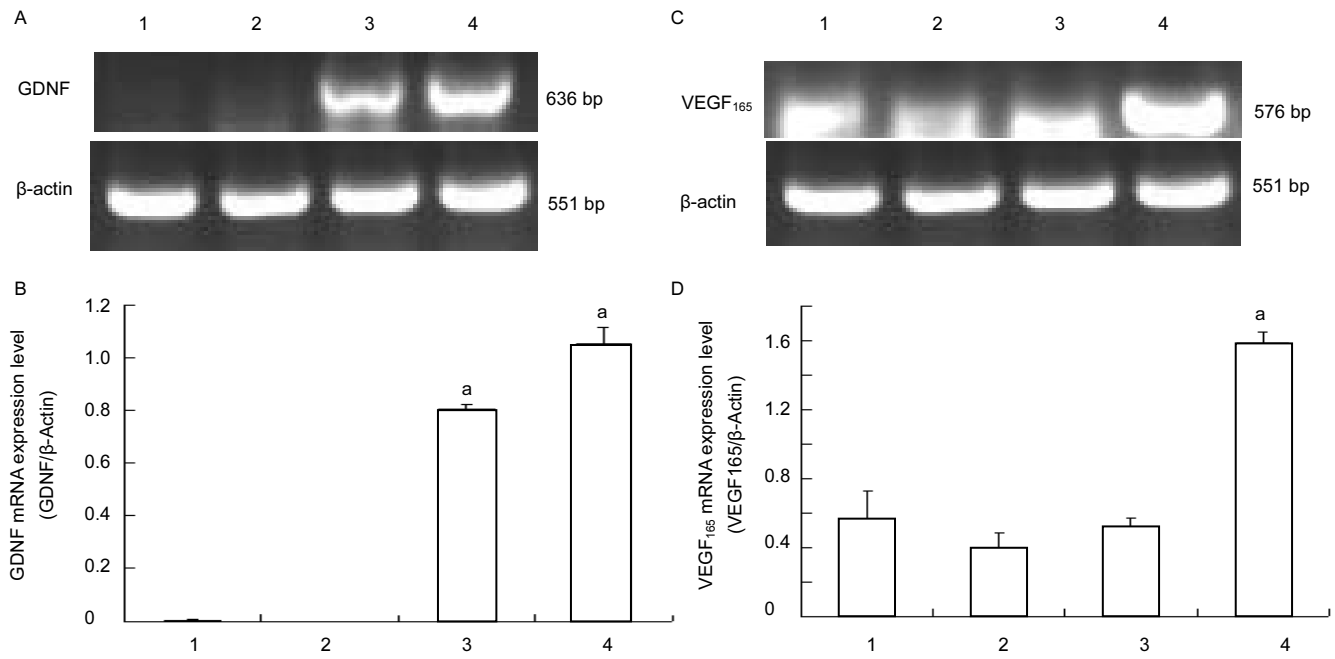

Figure 3 Expression of GDNF and VEGF$_{165}$ mRNA in HEK293 cells by RT-RCR

Note: (A, B) RT-PCR analysis of GDNF mRNA expression. (A) Lane 1: No transduced HEK293 cells; Lane 2: GFP Transduced HEK293 cells; Lane 3: GDNF transduced HEK293 cells; Lane 4: GDNF/VEGF$_{165}$ transduced HEK293 cells. (B) The mRNA expression level. The correspondence absorbance ratio of GDNF/β-actin mRNA. Data are expressed as mean±SD (n=3). $^aP < 0.01$, *vs*. no transduced or transduced by pIRES$_2$-EGFP. (C, D) RT-PCR analysis of VEGF$_{165}$ mRNA expression. (C) Lane 1: No transduced HEK293 cells; Lane 2: GFP transduced HEK293 cells; Lane 3: GDNF transduced HEK293 cells; Lane 4: GDNF/VEGF$_{165}$ transduced HEK293 cells. (D) The mRNA expression level. The correspondence absorbance ratio of VEGF$_{165}$/β-actin mRNA. Data are expressed as mean±SD (n=3). $^aP < 0.01$, *vs*. no transduced or transduced by pIRES$_2$-EGFP. GDNF: Glial cell line derived neurotrophic factor; VEGF: vascular endothelial growth factor.

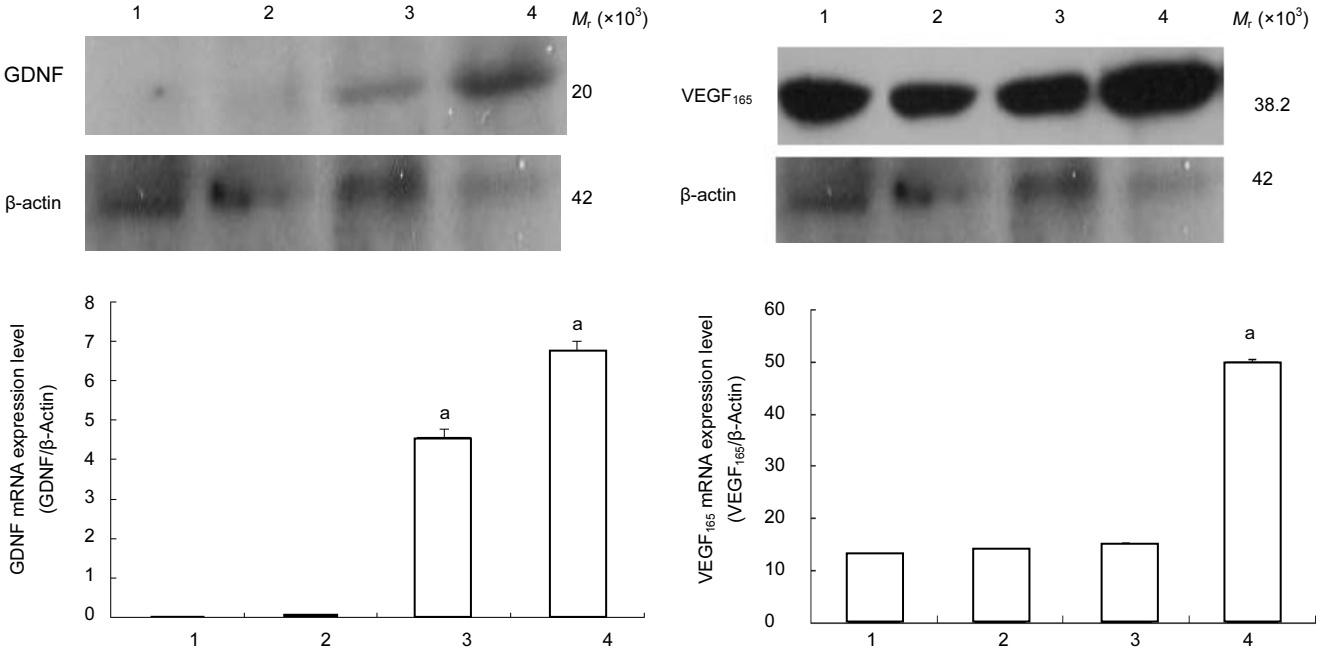

Figure 4 Expression of GDNF and VEGF$_{165}$ protein in HEK293 cells by western blot analysis

Note: (A, B) Western blot analysis of GDNF protein expression. (A) Lane 1: No transduced HEK293 cells; Lane 2: GFP transduced HEK293 cells; Lane 3: GDNF transduced HEK293 cells; Lane 4: GDNF/VEGF165 transduced HEK293 cells. (B) The correspondence absorbance ratio of GDNF/β-actin protein. Data are expressed as mean±SD (n=3). $^aP < 0.01$, *vs*. no transduced or transduced by pIRES$_2$-EGFP.

(C, D) Western blot analysis of VEGF$_{165}$ protein expression. (C) Lane 1: No transduced HEK293 cells; Lane 2: GFP transduced HEK293 cells; Lane 3: GDNF transduced HEK293 cells; Lane 4: GDNF/VEGF$_{165}$ transduced HEK293 cells.

(D) The protein expression level. The correspondence absorbance ratio of VEGF$_{165}$/β-actin protein. Data are expressed as mean±SD (n=3). $^aP < 0.01$, *vs*. no transduced or transduced by pIRES$_2$-EGFP. GDNF: Glial cell line derived neurotrophic factor; VEGF: vascular endothelial growth factor.

In recent years, VEGF has a direct effect on the growth of nerve cells, it can effectively reduce the spinal cord blood flow occlusion occurred after spinal cord injury[27]. Neural stem cells can effectively enhance the angiogenesis *in vivo* and blood-brain barrier density, improve the neurological function score and spinal cord tissue necrosis *in vitro* through the transfer of VEGF gene[28]. VEGF also can influence the apoptosis of neurons in spinal cord after injury[29]. Application of VEGF in treating diabetes, ischemic neuropathy, nerve regeneration, Parkinson's disease, Alzheimer's disease and multiple sclerosis, also show its neuroprotective effect[30].

In this study, we used the eukaryotic expression vector co-expressing GDNF and VEGF$_{165}$, and then transfect HEK293 cells with it. The results showed that HEK293 cells can highly express GDNF and VEGF$_{165}$, which provided the basis for the use of cells as a vector to transfer GDNF and VEGF$_{165}$ to spinal cord injury site.

In addition, GDNF and VEGF$_{165}$ genes were successfully inserted into a bicistronic eukaryotic expression vector and a plasmid pIRES$_2$-GDNF-VEGF$_{165}$ was constructed successfully. We plan to transfect this plasmid expressed by double genes into mesenchymal stem cells in the next experiment, in order to achieve the stable transfected cell lines. We surmised that double genes modified mesenchymal stem cells are better than single gene modified cells in promoting neuronal regeneration.

By gene recombination we successfully constructed eukaryotic expression vector of GDNF and VEGF$_{165}$, and these two genes can be co-expressed in HEK293 cells. Checking and analyzing the relative literatures, there is no study about gene therapy by using GDNF and VEGF$_{165}$ to promote differentiation of mesenchymal stem cells for spinal cord injury, so this experiment will provide new methods for the following animal experiments.

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人胶质细胞源性神经营养因子和血管内皮生长因子 165 双基因真核表达载体的构建与鉴定

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文章亮点：

1 在基因治疗中，联合应用多个具有协同作用的治疗基因通常可产生较单基因更为理想的效果。

2 实验将人胶质细胞源性神经营养因子和血管内皮生长因子 165 双基因成功插入到了双顺反子真核表达载体 pIRES$_2$(内部核糖体进入位点)中，采用一种简便和高效的方法成功构建了 pIRES$_2$-GDNF-VEGF$_{165}$ 双基因共表达载体。

关键词：

组织构建；组织工程；胶质细胞源性神经营养因子；血管内皮生长因子165；真核双表达载体；内部核糖体进入位点；转染；双PCR

主题词：

胶质细胞源性神经营养因子；血管内皮生长因子；载体蛋白质类；组织工程

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摘要

背景：人胶质细胞源性神经营养因子(glial cell line - derived neurotrophic factor，GDNF) 和血管内皮生长因子 165 (vascular endothelial growth factor 165，VEGF$_{165}$)在细胞分化过程中有重要作用。

目的：构建双基因共表达载体 pIRES$_2$-GDNF-VEGF$_{165}$ 并对其进行鉴定。

方法：采用 PCR 法从人外周血单个核细胞的基因组 DNA 中获取人胶质细胞源性神经营养因子基因，然后将人胶质细胞源性神经营养因子的 cDNA 片段插入到 pIRES$_2$-EGFP 多克隆位点构建成为 pIRES$_2$-GDNF-EGFP。人血管内皮生长因子 165 cDNA 片段是通过双 PCR 的方法从 pIRES$_2$-VEGF$_{165}$-EGFP 质粒中获取，接着将血管内皮生长因子 165 cDNA 片段以替换 EGFP 的方式插入 pIRES$_2$-BDNF-EGFP 中，最后构建成为含有内部核糖体进入位点(IRES)的 pIRES$_2$-GDNF-VEGF$_{165}$ 双基因共表达载体。通过双酶切和 DNA 测序方法对其鉴定，将重组的双基因共表达载体感染 HEK293 细胞，利用 RT-PCR 与 Western-blot 方法检测双基因的表达。

结果与结论：DNA 测序显示，提取的人胶质细胞源性神经营养因子和血管内皮生长因子 165 均与基因库报道序列一致，相对分子质量分别为 636 bp 和 576 bp。构建的 pIRES$_2$-GDNF-VEGF$_{165}$ 双基因共表达载体经 *Bgl* II/*Bam* HI 切出 GDNF 条带，经 B*am* HI/*Not* I 双酶切后切出 IRES-VEGF$_{165}$ 片段，经 *Bgl* II/*Not* I 双酶切后切出 GDNF-IRES-VEGF$_{165}$ 片段。RT-PCR 与 Western-blot 方法检测显示，此载体转染后，HEK293 细胞均能表达人胶质细胞源性神经营养因子和血管内皮生长因子 165 mRNA 和蛋白。说明实验成功构建了能表达人胶质细胞源性神经营养因子和血管内皮生长因子 165 的双基因真核表达载体。

作者贡献：第一作者与通讯作者负责课题设计、实验的实施、数据统计和论文的撰写与审核等，其他作者协助实验的实施和数据分析。

利益冲突：课题未涉及任何厂家及相关雇主或其他经济组织直接或间接的经济或利益的赞助。

伦理要求：健康人新鲜抗凝外周血获取征得供者同意并签署知情同意书。

学术术语：血管内皮生长因子 165-是分泌型可溶性蛋白，能直接作用于血管内皮细胞促进血管内皮细胞增殖，增加血管通透性。

作者声明：文章为原创作品，无抄袭剽窃，无泄密及署名和专利争议，内容及数据真实，文责自负。

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