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

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ABSTRACT. We used a simple and efficient method to construct the bicistronic eukaryotic expression vector pIRES2-NGF-VEGF$_{165}$. The nerve growth factor (NGF) gene was obtained from the genomic DNA of human peripheral blood mononuclear cells by polymerase chain reaction. The NGF cDNA fragment was inserted into the multiple cloning sites of the pIRES2-EGFP vector to generate the bicistronic eukaryotic expression plasmid pIRES2-NGF-EGFP. The vascular endothelial growth factor 165 (VEGF$_{165}$) gene was obtained from the pIRES2-VEGF$_{165}$-EGFP plasmid by polymerase chain reaction. Next, the VEGF$_{165}$ cDNA fragment was cloned into pIRES2-NGF-EGFP in place of enhanced green fluorescent protein creating the plasmid pIRES2-NGF-VEGF$_{165}$. pIRES2-NGF-VEGF$_{165}$ was transfected into HEK293 cells and reverse transcription-polymerase chain reaction and Western blot analysis were used to test the co-expression of double genes. The NGF and VEGF$_{165}$ genes were cloned and the DNA was sequenced, which revealed that NGF and VEGF$_{165}$ were consistent with the sequence recorded in GenBank. Restriction analysis showed that the NGF and VEGF$_{165}$ genes were inserted into the expression vector pIRES2-EGFP. Transfection of pIRES2-NGF-

VEGF$_{165}$ into HEK293 cells resulted in expression of the double gene at the mRNA and protein levels. The NGF and VEGF$_{165}$ coexpression plasmid provides a novel expression system, enabling further study of the functions of the NGF and VEGF$_{165}$ genes.

Key words: Bicistronic eukaryotic expression vector; VEGF$_{165}$; Nerve growth factor; Internal ribosome entry site

INTRODUCTION

Recent experiments have shown that neurotrophic factors also promote axonal growth; when combined with stem cell grafts, these factors can further amplify axonal extension after injury (Ziv-Polat et al., 2012). Continued preclinical development of these approaches for neural repair may result in the development of treatment strategies for human injury. Mesenchymal stem cells (MSCs) are thought to be a good source of stem cells because of their tremendous self-renewal capacity and multi-lineage differentiation potential (Huang et al., 2010). Curative effects have been observed in animal experiments using MSCs to treat ischemic brain injury (Wojciech et al., 2012). Human bone marrow cells have been extensively used to treat hematologic diseases (Senn and Pinkerton, 2012). Moreover, non-hematopoietic stem cells, such as MSCs, can differentiate into mature osteocytes, chondrocytes, and adipocytes (Liu et al., 2009). Bone marrow cells differentiate into mature neurons or glial cells when induced under experimental conditions (Ribeiro et al., 2011). These findings indicate that bone marrow cells can be used therapeutically in patients with neurological diseases, which would circumvent the ethical problems surrounding the use of embryonic stem cells.

Enhancing the proliferation and orientational differentiation ability of stem cells by gene modification has received increasing attention in the tissue engineering field. Nerve growth factor (NGF) (Esposito et al., 2011), vascular endothelial growth factor (VEGF) (Widenfalk et al., 2003), brain-derived neurotrophic factor, or the derived scaffolds or vectors constructed based on these factors have been widely examined in experiments and gradually applied clinically. In this study, we constructed the bicistronic eukaryotic expression vector pIRES2-NGF-VEGF$_{165}$ using a simple and efficient method to enable further study of the functions of the NGF and VEGF$_{165}$ genes.

MATERIAL AND METHODS

Materials

The study was approved by the ethics review board of Xinxiang Medical University. pIRES$_2$-EGFP was obtained from the Beijing Tian ENZE Gene Technology Co., Ltd., Beijing, China. pIRES2-VEGF$_{165}$-EGFP was obtained from Stem Cell and Biotherapy Technology Research Center of Xinxiang Medical University. Human peripheral blood mononuclear cells were provided by the first author Dr. Bingnan Li. T4 DNA ligase, Prime STAR Max DNA Polymerase, *Xho*I, *Bam*HI, *Not*I, and *Bst*XI restriction enzymes the total RNA extraction kit, reverse transcription kit, high-purity gel extraction kit, and DNA marker were from Takara Biotechnology (Dalian) Co., Ltd. (Shiga, Japan).

Primer design

The NGF (NM_002506.2) and $VEGF_{165}$ gene sequences (NM_001025368.2) in GenBank served as the templates and were used to design primers.

The coding sequence of human NGF gene fragment was present in exon 3 of the gene. Two primers were designed to amplify human NGF. An *Xho*I restriction enzyme site and protective base were added to the upstream primer (5'-CCCTCGAGATGTCCATGTTGTTC-3'), and *Bam*HI restriction enzyme site and protective base were added to the downstream primer (5'-CGGGATCCTCAGGCTCTTCTCCACAG-3'). The length of the amplified fragment was 726 base pairs (bp). Two primer pairs were designed to amplify the human VEGF. The forward-long primer (5'-AACCATGAACTTTCTGCTGTCTTGGGTGCATT-3') was 4 bases longer than the forward-short (5'-ATGAACTTTCTGCTGTCTTGGGTGC-3') at the 5' end. The primer reverse-long (5'-GGCCGCTCACCGCCTCGGCTTG-3') was longer than the reverse-short (5'-GCTCACCGCCTCGGCTTGTCA-3'). The underlined bases in forward primers indicate the *Bst*XI site, and in reverse primers the *Not*I site, which were used to assemble the *Bst*XI and *Not*I sticky ends. The length of amplified fragment was 576 bp.

Construction of pIRES2-NGF-EGFP

The genomic DNA of human peripheral blood MNCs severed as the template. The NGF primer was used to amplify NGF gene. The coding sequence of human NGF gene fragment was present only in exon 3 of the gene. A total of 20 µL polymerase chain reaction (PCR) system was added, including 0.5 µL (10 ng) genomic DNA, 10 µL 2X Prime STAR Max DNA Polymerase, RNase-free water 7.5 µL, and 2 µL of each upstream and downstream primers. The reaction conditions were: 95°C for 5 min, 98°C for 10 s, 55°C for 5 s, and 72°C for 5 s for 30 cycles; a temperature of 72°C was maintained for 5 min. The PCR product was purified using the PCR purification kit, and the PCR product and plasmid pIRES2-EGFP were digested by *Xho*I and *Bam*HI. After digestion, the PCR product was purified using the PCR purification kit. The DNA fragment was inserted into the plasmid by T4 DNA ligase in a total volume of 20 µL, including 2 µL $pIRES_2$-EGFP, 8 µL cDNA (NGF), and 0.2 µL T4 DNA ligase added at 22°C for 30 min. The DNA fragment was transformed into *Escherichia coli* DH5α and plated on an LB plate (kan^r). The plate was placed in an incubator at 37°C for 16 h. Single colonies were shaken for 12-16 h at 37°C at 225 rpm. The plasmid was extracted and identified using *Xho*I and *Bam*HI double enzyme digestion. The recombinant plasmid pIRES2-NGF-EGFP was obtained.

Construction of pIRES2/NGF-$VEGF_{165}$

A simple and efficient method was developed for directional cloning of PCR products without restriction enzyme digestion of the amplified sequence. Two pairs of primers were designed in which parts of 2 restriction enzyme recognition sequences had been integrated, and the primers were used for 2 parallel PCRs. The PCR products were mixed, heat denatured, and re-annealed to generate hybridized DNA fragments bearing sticky ends compatible with the restriction enzymes. This method is particularly useful when it is necessary to use a restriction enzyme, but there is an additional internal restriction site within the amplified sequence, or when there are problems caused by end sensitivity of restriction enzymes.

PCR amplification of VEGF$_{165}$

Two parallel PCRs were set up using either forward-short/reverse-long or forward-long/reverse-short primer pairs. The VEGF$_{165}$ gene was obtained from the pIRES2-VEGF$_{165}$-EGFP plasmid by PCR. Both amplifications were subjected to 30 cycles at 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min, followed by a 5 min extension at 72°C using Prime STAR Max DNA Polymerase. The DNA fragments expected in the 4 PCR products were purified separately using a DNA gel extraction kit and quantified using a Nano Drop2000UV-Vis spectrophotometers (Wilmington, DE, USA).

Cloning of PCR products

pIRES2-NGF-EGFP was digested with *Bst*XI and *Not*I, followed by phenol:chloroform extraction and ethanol precipitation. Each pooled VEGF$_{165}$ PCR product was mixed, denatured at 94°C for 4 min, and re-annealed at 65°C for 2 min. For the ligation reaction, the annealed VEGF and linearized pIRES2-NGF-EGFP (molar ratio equal to 4:1) were incubated with T4 DNA ligase at 16°C for 3 h. The ligation products were used to transform *E. coli* DH5α $CaCl_2$ competent cells following a standard method. Screening of the transformants was performed by *Bst*XI plus *Not*I digestion following alkaline lysis plasmid preparation, and the recombinants were confirmed by DNA sequencing (Figure 1).

In vitro transduction of HEK293 cells

HEK293 cells were maintained in Dulbecco's modified Essential Medium containing 10% newborn bovine serum (Hangzhou Sijiqing Biological Products, Hangzhou, China) and 100 μg/mL penicillin/streptomycin. Cells were maintained in a humidified environment at 37°C and 5% CO_2. Cell viability was monitored using the trypan blue exclusion method. The viability was over 95% in all experiments. Cells were seeded at a density of 5 x 10^5 cells/well on a 6-well tissue culture plate and cultured for 24 h to 60-80% confluence. HEK293 cells were either mock-infected or infected with the pIRES2-EGFP, pIRES2-NGF/EGFP, or pIRES$_2$-NGF/VEGF$_{165}$ vector using Lipofectamine™ 2000 for 2 h at 37°C 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, the cells were observed under an inverted fluorescent microscope. The expression of the NGF and VEGF$_{165}$ genes was analyzed by reverse transcription (RT-PCR).

To detect the NGF and VEGF$_{165}$ mRNA expression levels in HEK293 cells that had been either mock-infected or infected with the pIRES2-EGFP, pIRES2-NGF/EGFP, or pIRES2-NGF/VEGF$_{165}$ vectors, after 3 days the NGF and VEGF$_{165}$ expression was primarily assessed by RT-PCR. Total RNA and reverse transcription were conducted as described above. PCR amplification of the sequences from harvested cDNAs was conducted using the following primers: NGF, primer1 (forward): 5'-CCCTCGAGATGTCCATGTTGTTC-3' and primer2 (reverse): 5'-CGGGATCCTCAGGCTCTTCTCCACAG-3'; VEGF$_{165}$ primer3 (forward): 5'-AACCATGAACTTTCTGCTGTCTTGGGTGCATT-3' and primer4 (reverse): 5'-GGCCGCTCACCGCCTCGGCTTG-3'; β-actin primer5 (forward): 5'-AGCGGGAAATCGTGCGTGAC-3' and primer6 (reverse): 5'-CAAGAAAGGGTGTAACGCAACTA-3'.

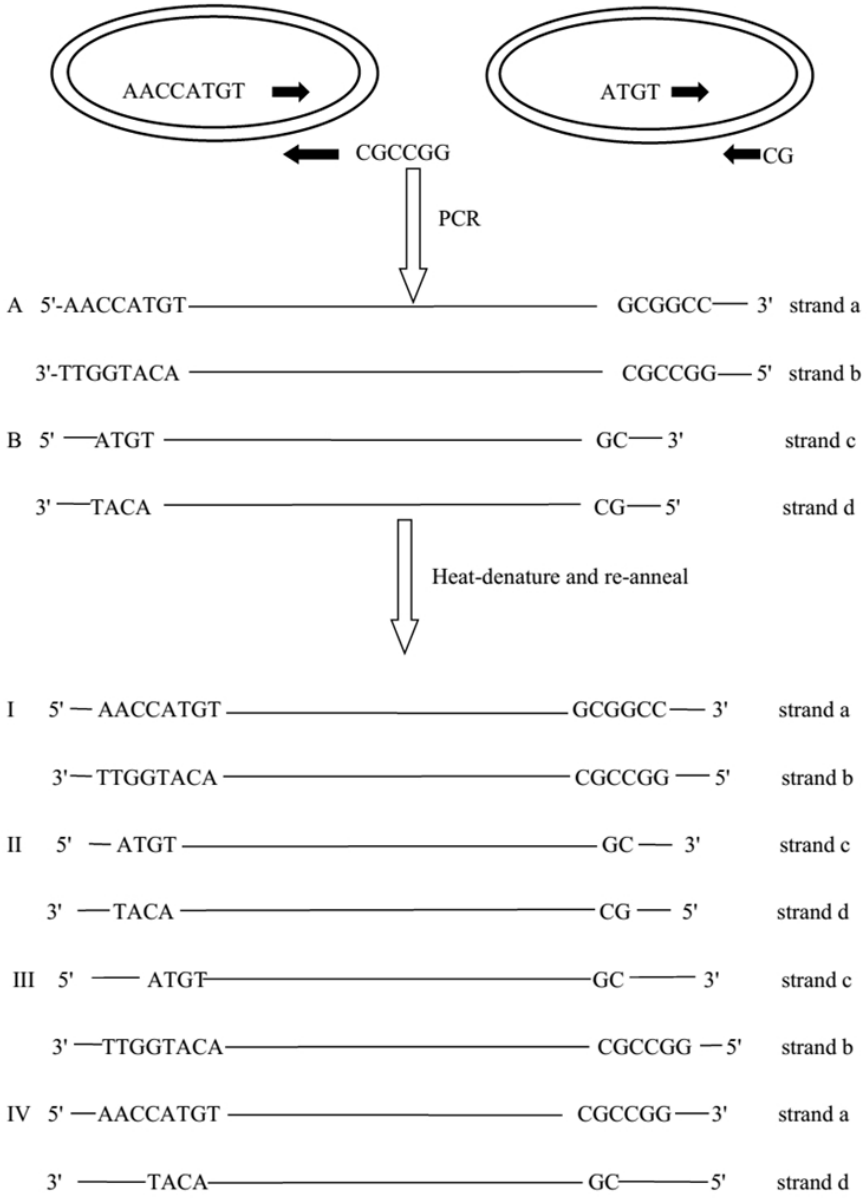

Figure 1. Outline of twin PCR. Two parallel PCRs were set up using either forward-long/reverse-long (PCR products A) or forward-short/reverse-short primer pairs (PCR products B), with the pIRES2-VEGF$_{165}$-EGFP plasmid (double ring indicates this plasmid in this figure) as templates. Equimolar of the 2 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 were generated via random complementary pairing, including molecules I, II, III, and IV. Two of the products, I and II, were blunt-ended, while the other 2 were sticky-ended. The molecule III has a 5' *Bst*XI sticky end and a 3' *Not*I sticky end, which allows the fragment to be cloned directionally into pIRES2-NGF-EGFP that has been digested with *Bst*XI and *Not*I.

A standard Western blotting protocol was used to detect NGF and VEGF$_{165}$ protein expression in HEK293 cells that had been either mock-infected or infected with the pIRES2-EGFP, pIRES2-NGF/EGFP, or pIRES2-NGF/VEGF165 vectors three days later. The culture supernatants of infected HEK293 cells without serums were collected and subjected to western blotting analysis using anti-NGF and anti-VEGF$_{165}$ antibodies. The 20 μg protein extracted from transduced HEK293 cells was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing and denaturing conditions and transferred to polyvinylidene fluoride membranes. The membranes were incubated in a 1:500 dilution polyclonal rabbit anti-NGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 1:500 dilution 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 Tris-buffered saline 3 times (10 min per wash) followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000) at room temperature for 2 h. The membrane was washed 3 times with Tris-buffered saline for 15 min each and exposed to X-ray film to observe the results. The signal was quantified using the Gel-Pro analyzer software.

Statistical analysis

Results are reported as means ± SD recorded from 3 experiments. The significance of differences was determined by one-way analysis of variance.

RESULTS

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

The NGF gene was obtained from the genomic DNA of human peripheral blood mononuclear cells (MNCs) by PCR. The size of NGF gene was 726 bp. The VEGF$_{165}$ gene was obtained from pIRES2-VEGF$_{165}$-EGFP plasmid by PCR. The size of VEGF$_{165}$ gene was 576 bp (Figure 2A).

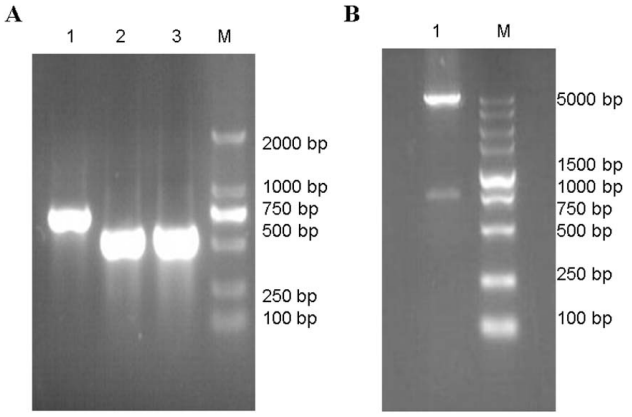

Figure 2. A. Amplification of NGF and VEGF$_{165}$. *Lane M* = marker; *lane 1* = NGF gene; *lane 2* = VEGF$_{165}$ (forward-short/reverse-short primer pairs) gene; *lane 3* = VEGF$_{165}$ (forward-long/reverse-long primer pairs) gene. **B.** Identification of plasmid pIRES2-NGF-EGFP. *Lane M* = marker; *lane 1* = pIRES2-NGF-EGFP cut by *Xho*I and *Bam*HI double digestion.

Identification of plasmid pIRES2-NGF-EGFP

The plasmid pIRES$_2$-NGF-EGFP was cut by *Xho*I and *Bam*HI double digestion. A gene fragment of 726 bp was obtained, which agreed with the known size of the NGF gene (Figure 2B).

Identification of plasmid pIRES2-NGF-VEGF$_{165}$

The plasmid pIRES$_2$-NGF-VEGF$_{165}$ was cut by *Xho*I and *Bam*HI double digestion, resulting in a fragment of 726 bp. This indicated that the NGF gene had been inserted into the plasmid pIRES2-NGF-VEGF$_{165}$. The sequence of the plasmid pIRES2- NGF-VEGF$_{165}$ was in accordance with gene sequence in GenBank (Figure 3).

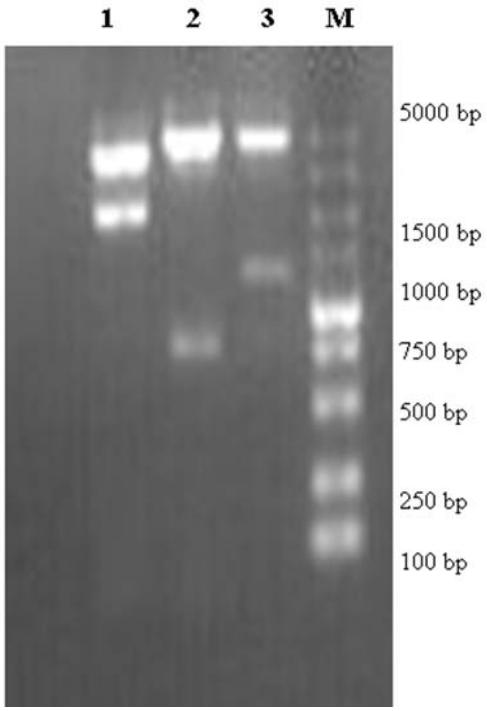

Figure 3. Identification of plasmid pIRES2-NGF-VEGF$_{165}$. *Lane M* = marker; *lane 1* = pIRES2-NGF-VEGF$_{165}$ cut by *Xho*I and *Not*I; *lane 2* = pIRES2-NGF-VEGF$_{165}$ cut by *Xho*I and *Bam*HI; *lane 3* = pIRES2-NGF-VEGF$_{165}$ cut by *Bam*HI and *Not*I.

RT-PCR analysis of NGF and VEGF$_{165}$ expression

To illustrate mRNA expression by pIRES2-EGFP-, pIRES2-NGF/EGFP-, and pIRES2-NGF/VEGF$_{165}$-transduced HEK293 cells, we evaluated the expression of NGF and VEGF$_{165}$ by RT-PCR analysis. RT-PCR was performed using NGF-specific primers and the

β-actin sequence as an internal standard. Green fluorescent protein (GFP) expression was monitored in pIRES2-EGFP- and pIRES2-NGF/EGFP-transduced HEK293 cells by inverted fluorescence microscopy. Expression of NGF mRNA was higher in pIRES2-NGF/EGFP- and pIRES2-NGF/VEGF$_{165}$-transduced HEK293 cells than in pIRES2-EGFP-transduced HEK293 cells or in negative controls (Figure 4A and B). Expression of VEGF$_{165}$ mRNA was higher in pIRES2-NGF/VEGF$_{165}$-transduced HEK293 cells than in the other 3 cells (Figure 4C and D). These results demonstrated that the NGF and VEGF$_{165}$ was successfully introduced into HEK293 cells containing pIRES2-NGF/EGFP and pIRES2-NGF/VEGF$_{165}$. After the HEK293 cells were transduced with pIRES2-NGF/EGFP, they were passaged continually and then the mean percentage of GFP expression was monitored under fluorescence microscopy. There was no decrease in GFP fluorescence, illustrating the maintenance of transgenic expression in the transduced cells.

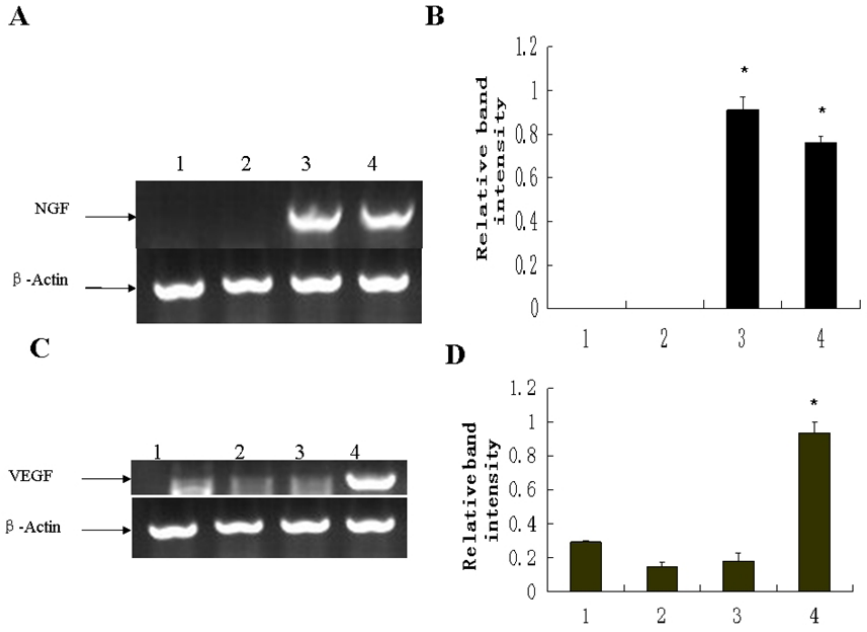

Figure 4. A. B. RT-PCR analysis of NGF expression. NGF = 726 bp, β-actin = 551 bp. **A.** *Lane 1* = non-transduced HEK293 cells; *lane 2* = GFP-transduced HEK293 cells; *lane 3* = NGF-transduced HEK293 cells; *lane 4* = NGF/VEGF$_{165}$ -transduced HEK293 cells. **B.** mRNA expression level. Corresponding OD ratio of NGF/β-actin. Data are reported as means ± SD (N = 3), compared with non-transduced or transduced by pIRES2-EGFP. *P < 0.01. **C. D.** RT-PCR analysis of VEGF$_{165}$ expression. VEGF$_{165}$ = 576 bp, β-actin = 551 bp. **C.** *Lane 1* = non-transduced HEK293 cells; *lane 2* = GFP-transduced HEK293 cells; *lane 3* = NGF-transduced HEK293 cells; *lane 4* = NGF/VEGF$_{165}$-transduced HEK293 cells. **D.** mRNA expression level. Corresponding OD ratio of VEGF$_{165}$/β-actin. Data are reported as means ± SD (N = 3), compared with non-transduced HEK293 cells or transduced with pIRES2-EGFP. *P < 0.01.

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

To illustrate protein expression by pIRES2-EGFP-, pIRES2-NGF/EGFP-, and pIRES2-NGF/VEGF$_{165}$-transduced HEK293 cells, we evaluated the expression of NGF and VEGF$_{165}$ by Western blot analysis. Seventy-two hours after transduction, we collected the cul-

ture supernatants of infected HEK293 cells without serum, which were processed for Western blotting using anti-NGF and anti-$VEGF_{165}$ antibodies. β-actin was used as an internal standard. Exogenous NGF protein was strongly expressed in pIRES2-NGF/EGFP- and pIRES2-NGF/$VEGF_{165}$-transduced HEK293 cells, but the pIRES2-EGFP-transduced HEK293 cells and non-transduced expression of endogenous NGF were very low (Figure 5A and B). As shown above, Western blot analysis also revealed that exogenous $VEGF_{165}$ protein was strongly expressed in pIRES2-NGF/$VEGF_{165}$-transduced HEK293 cells, and the pIRES2-EGFP, pIRES2-NGF/EGFP-transduced HEK293 cells expressed very low levels of endogenous $VEGF_{165}$ (Figure 5C and D). These results also demonstrated that NGF and $VEGF_{165}$ had been successfully introduced into HEK293 cells by pIRES2-NGF/$VEGF_{165}$.

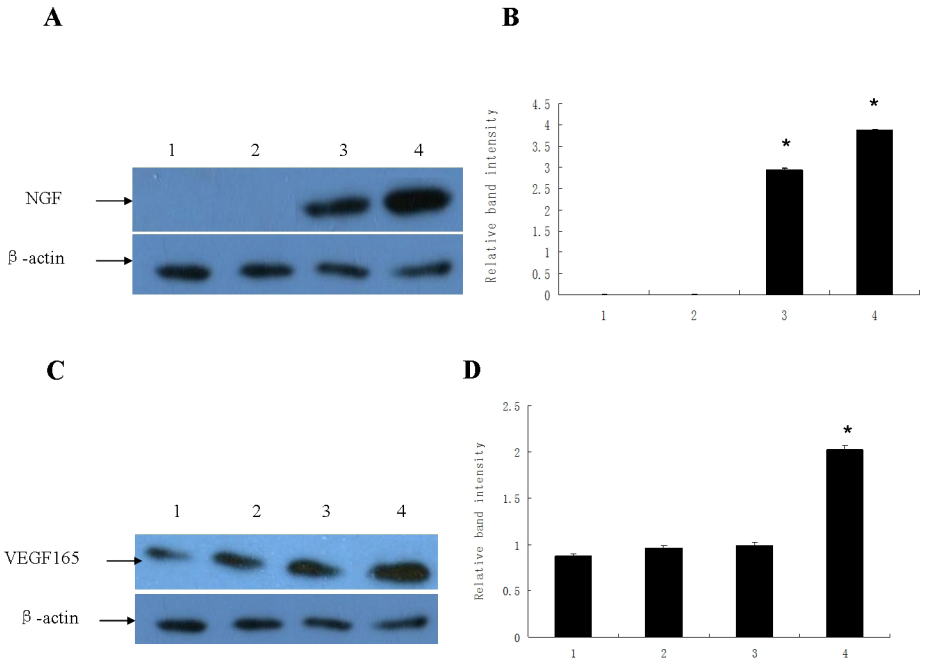

Figure 5. A. B. Western blot analysis of NGF expression. NGF = 28 kDa, β-actin = 42 kDa. **A.** *Lane 1* = non-transduced HEK293 cells; *lane 2* = GFP-transduced HEK293 cells; *lane 3* = NGF-transduced HEK293 cells; *lane 4* = NGF/$VEGF_{165}$-transduced HEK293 cells. **B.** Corresponding OD ratio of NGF/β-actin. Data are reported as means ± SD (N = 3), compared with non-transduced or transduced with pIRES2-EGFP. *P < 0.01. **C. D.** Western blot analysis of $VEGF_{165}$ expression. $VEGF_{165}$ = 38.2 kDa, β-actin = 42 kDa. **C.** *Lane 1* = non-transduced HEK293 cells; *lane 2* = GFP-transduced HEK293 cells; *lane 3* = NGF-transduced HEK293 cells; *lane 4* = NGF/$VEGF_{165}$-transduced HEK293 cells. **D.** Protein expression level. Corresponding OD ratio of $VEGF_{165}$/β-actin. Data are reported as means ± SD (N = 3), compared with non-transduced or transduced with pIRES2-EGFP, pIRES2-NGF/EGFP. *P < 0.05.

DISCUSSION

To improve the cloning efficiency and simplify the cloning procedure, we developed twin PCR to create insert DNA with single-stranded overhangs at the ends that were complementary to restriction enzyme-digested vector (Xie and Xie, 2011). As shown in Figure 1, the PCR products reassembled via complementary pairing after heat-denaturing and re-annealing.

Four types of molecules were created in equal proportions, 2 of which were blunt-ended and 2 were sticky-ended. Theoretically, half of the sticky-ended products should be suitable for ligation. Using the twin PCR strategy, the hybridized PCR products flanked with various types of sticky ends that are compatible with nearly all restriction enzymes can be generated based solely on primer design and PCR amplification. Because no restriction enzyme digestion is required for the PCR products, twin PCR overcomes the limitations caused by end sensitivity of restriction enzymes, and the internal restriction sites within the amplified sequences will not limit the selection of cloning sites. This is particularly useful for cloning long PCR products, which may contain several restriction enzymes sites.

In this study, VEGF$_{165}$ was directly inserted into pIRES2-NGF-EGFP at the *Bst*XI and *Not*I sites. Efficient and economical enzymes, such as *Bst*XI and *Not*I, can be used for vector digestion, limiting the number of restriction enzymes necessary for cloning. Additionally, our cloning method has other advantages. It is very simple that the whole procedure, including PCR, enzyme digestion, ligation, and transformation can be completed in 8 h, saving time, cost, and labor. In addition, inserts of interest can be amplified using proof-reading DNA polymerase, providing a faithful guarantee of the PCR products. These advantages make this technique universal for the directional cloning of PCR products.

MSCs are a population of self-renewing, multipotent cells that can differentiate along several committed phenotypes, including osteogenic, chondrogenic, and adipogenic lineages in response to stimulation by multiple environmental factors (Bianco et al., 2008). Various cytokines and transcription factors play an important role in this procedure.

NGF is a potent stimulus of sensory axon growth after injury (Lu et al., 2008). Continuous infusion of NGF into the dorsal spinal cord rostral to a peripheral nerve graft promotes the growth of sensory axons from the graft into the dorsal column white matter (Franz et al., 2012). For example, after peripheral dorsal root crush lesions, only 3% of sensory axons were found to re-enter the spinal cord through the dorsal root entry zone. This proportion was significantly increased to 37% after 14 days of continuous NGF infusion through catheters placed in the dorsal spinal cord (Jones et al., 2001). These regenerating axons penetrate spinal cord white matter and grow up to 3 mm past the dorsal root entry zone (Duffy et al., 2009). Additional experiments confirmed this NGF-driven regrowth of injured sensory fibers into the spinal cord and revealed the regenerating axons as positive for calcitonin gene-related peptide, a marker for small diameter, unmyelinated peptidergic axons (Lykissas et al., 2007). This study confirmed using electro-physiology-based experiments that postsynaptic potentials can be evoked in the dorsal horn of the spinal cord after peripheral stimulation of injured axons. Functional improvements were also observed through noxious behavioral testing. In further experiments, NGF was delivered to the injured spinal cord using grafts of primary fibroblasts that were genetically modified to produce and secrete NGF into sites of spinal cord injury (Jones et al., 2003). In addition to confirming robust sensitivity of injured calcitonin gene-related peptide-expressing sensory nociceptive axons to NGF, these experiments showed that an NGF source within the spinal cord also attracted the growth of coerulospinal axons and ventral motor axons (Kamei et al., 2007).

VEGF, originally identified because of its ability to induce the growth of new blood vessels, can protect neurons from damage induced by ischemia and trauma (Rosenstein et al., 2010). Intramuscular injection of a plasmid coding for VEGF prevented the development of diabetic neuropathy in rodents and rabbits (Yoon et al., 2009). VEGF was previously shown to display neuroprotective effects following ischemia, suggesting that VEGF may be applied as a

neuroprotective agent for treating other neurological diseases (Fujiki et al., 2010). The role of VEGF in diabetic neuropathy is complex. VEGF levels were found to be increased in Schwann cells of rats with diabetic neuropathy, potentially in response to nerve hypoxia (Chattopadhyay et al., 2005). Systemic expression of VEGF achieved by the intramuscular gene transfer of naked DNA encoding VEGF restored large- and small-fiber peripheral nerve function in a rat model of diabetic neuropathy and a rabbit model of alloxan-induced diabetic neuropathy (Potenza et al., 2011). In a human trial, a plasmid encoding $VEGF_{165}$ was injected into the muscle of patients with leg ischemia, resulting in significant clinical improvement in neuropathic symptoms, nerve conduction measures, and vibration threshold (Shyu et al., 2003).

In this study, the NGF and $VEGF_{165}$ genes were inserted into a bicistronic eukaryotic expression vector. The plasmid pIRES2-NGF-$VEGF_{165}$ was successfully constructed. In future studies, the plasmid can be transfected into MSCs and the NGF and $VEGF_{165}$ genes can be expressed in these cells. Combined expression of these genes may have more significant effects on neuron regeneration than each single gene alone.

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