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Construction and identification of pIRES$_2$-VEGF$_{165}$-NT-3 bicistronic eukaryotic expression vector

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ABSTRACT. We used a simple and efficient method to construct the bicistronic eukaryotic expression vector pIRES$_2$-VEGF$_{165}$-NT-3. The neurotrophin-3 (*NT-3*) gene was obtained from the genomic DNA of human peripheral blood mononuclear cells by polymerase chain reaction. The *NT-3* cDNA fragment was cloned into the pIRES$_2$-VEGF$_{165}$-EGFP vector in place of enhanced green fluorescent protein (EGFP) to create the plasmid pIRES$_2$-VEGF$_{165}$-NT-3. Next, pIRES$_2$-VEGF$_{165}$-NT-3 was transfected into HEK293 cells, and reverse transcription-polymerase chain reaction and Western blotting were used to test co-expression of the double genes. The vascular endothelial growth factor 165 (*VEGF$_{165}$*) and *NT-3* genes were cloned; DNA sequencing analysis demonstrated that the *VEGF$_{165}$* and *NT-3* sequences were the same as those recorded in GenBank. Restriction analysis indicated that the *VEGF$_{165}$* and *NT-3* genes were correctly inserted into the expression vector pIRES$_2$-EGFP. The double gene was expressed at both the mRNA and protein levels. The *VEGF$_{165}$* and *NT-3* co-expression plasmid was successfully

constructed, providing a novel expression system for further study of the functions of the *VEGF$_{165}$* and *NT-3* genes.

Key words: Bicistronic eukaryotic expression vector; Internal ribosome entry site; Neurotrophin-3; Vascular endothelial growth factor 165

INTRODUCTION

Traumatic spinal cord injury affects many people and can result in severe neurological damage (Domingo et al., 2012). Recovery from central nervous system (CNS) injury is difficult because of the limited ability of the injured CNS to regenerate lost cells, replace disrupted myelin, and reestablish functional neural connections (Zhao et al., 2013). One strategy for increasing axonal regeneration involves the transplantation of stem cells into the injured spinal cord (Sahni and Kessler, 2010).

Mesenchymal stem cells (MSCs) are thought to be useful as stem cells because of their tremendous self-renewal and multi-lineage differentiation potential (Huang et al., 2010). Some curative effects have been observed in animal experiments using MSCs to treat ischemic brain injury (Maksymowicz et al., 2012). Human bone marrow cells have been used to treat hematologic diseases (Senn and Pinkerton, 1972). Moreover, non-hematopoietic stem cells, such as MSCs, can differentiate into mature osteocytes, chondrocytes, and adipocytes (Liu et al., 2009). A previous study found that 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 applied therapeutically in patients with neurological diseases, which would also obviate the ethical problems associated with the use of embryonic stem cells.

Enhancement of the proliferation and orientation differentiation ability of stem cells by gene modification has been widely studied in tissue engineering. Vascular endothelial growth factor 165 (VEGF$_{165}$) (Widenfalk et al., 2003), neurotrophin-3 (NT-3) (Schnell et al., 2011), brain-derived neutrophic factor, or the derived scaffolds or vectors constructed based on these factors have been widely used in experimental studies and gradually applied in the clinical setting. The goal of this study was to construct a bicistronic eukaryotic expression vector, pIRES$_2$-VEGF$_{165}$-NT-3, using a simple and efficient method to enable further study on the functions of the *VEGF$_{165}$* and *NT-3* genes.

MATERIAL AND METHODS

Materials

pIRES$_2$-EGFP was obtained from Beijing Tian ENZE Gene Technology Co., Ltd. (Beijing, China). pIRES$_2$-VEGF$_{165}$-EGFP was procured from the Stem Cell and Biotherapy Technology Research Center of Xinxiang Medical University. Human peripheral blood mononuclear cells (MNCs) were obtained from healthy donors. T4 DNA ligase, Prime STAR Max DNA polymerase, *Eco*RI, *Bam*HI, *Not*I, *Bst*XI, restriction enzyme, total RNA extraction kit, reverse transcription kit, high-purity gel extraction kit, and DNA marker were obtained from Takara Biotechnology Co., Ltd. (Dalian, Shiga, Japan).

Primer design

The *VEGF*$_{165}$ gene sequence (NM_001025368.2) and *NT-3* gene sequence (GenBank: AF486837.1) in GenBank served as templates for designing the primers. Two primers were designed to amplify *VEGF*$_{165}$. An *Eco*RI restriction enzyme cut site and protective base was added to the upstream primer (5'-CGGAATTCATGAACTTTCTGCTGTCTTGGGTGC-3'), while a *Bam*HI restriction enzyme cut site and protective base was added to the downstream primer (5'-CGGGATCCTCACCGCCTCGGCTTGTCA-3'). The length of the amplified fragment was 576 base pairs (bp).

Two primer pairs were designed to amplify human *NT-3*. The forward-long primer (5'-AACCATGTCCATCTTGTTTTATGTGATATTTC-3') was 4 bases longer than the forward-short primer (5'-ATGTCCATCTTGTTTTATGTGATATTTCTCGC-3') at the 5' end. The same was true for the reverse-long (5'-GGCCGCTCATGTTCTTCCGATTT-3') compared to the reverse-short primer (5'-GCTCATGTTCTTCCGATTTTTCTCGAC-3'). The underlined bases in forward and reverse primers indicate the introduced *Bst*XI and *Not*I sites, respectively, which were used to assemble the *Bst*XI and *Not*I sticky ends. The length of the amplified fragment was 774 bp.

Construction of pIRES$_2$-VEGF$_{165}$-NT-3

A simple and efficient method was developed for directional cloning of polymerase chain reaction (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 were 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 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 the end sensitivity of restriction enzymes.

PCR amplification of *NT-3*

Two parallel PCRs were set up using either the forward-long/reverse-long or forward-short/reverse-short primer pairs, with the genomic DNA of human peripheral blood MNCs as templates. Both amplification reactions were subjected to 30 cycles of 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 expected DNA fragments in the 4 PCR products were purified separately using a DNA gel extraction kit and quantified using a NanoDrop 2000 UV-Vis Spectrophotometer (NanoDrop; Wilmington, DE, USA).

Cloning of PCR products

pIRES$_2$-VEGF$_{165}$-EGFP was digested with *Bst*XI and *Not*I, followed by phenol:chloroform extraction and ethanol precipitation. Each of the pooled *NT-3* PCR products amplified using Vent polymerase were mixed, denatured at 94°C for 4 min, and re-an-

nealed at 65°C for 2 min. For the ligation reaction, the annealed *NT-3* and linearized pIRES$_2$-VEGF$_{165}$-EGFP (molar ratio equal to 4:1) were incubated with T4 DNA ligase at 16°C for 3 h. For TA cloning, the 2 PCR products amplified by using EX Taq polymerase were mixed in equimolar ratios and ligated into pMD18-T according to manufacturer instructions. The 2 ligation products were used to transform *Escherichia coli* DH5α $CaCl_2$ competent cells following the standard method. Screening of the transformants was performed by *Bst*XI and *Not*I digestion following alkaline lysis plasmid preparation. Recombinant plasmid sequences were confirmed by DNA sequencing (Figure 1).

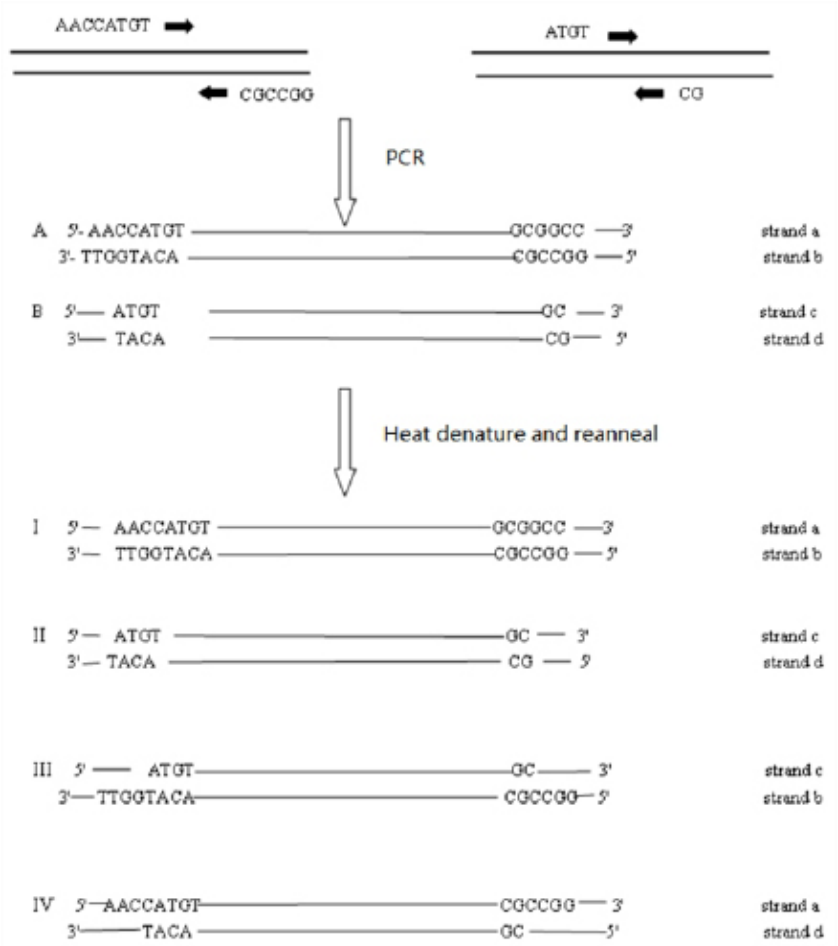

Figure 1. Outline of twin PCR method. Two parallel PCRs were set up using either forward-long/reverse-long (PCR products **A**) or forward-short/reverse-short primer pairs (PCR products **B**), with the genomic DNA of human peripheral blood mononuclear cells as templates. Equimolar amounts of the 2 PCR products **A** and **B** were mixed, heat denatured, and re-annealed to generate hybridized DNA fragments. Four types of double-stranded DNA molecules of equal proportions are generated via random complementary pairing, including molecules I, II, III, and IV. Two of these, I and II, were blunt-ended, while III and IV were sticky-ended. Molecule III possessed a 5' *Bst*XI sticky end and a 3' *Not*I sticky end, allowing the fragment to be cloned directionally into pIRES$_2$-VEGF$_{165}$-EGFP that had been 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 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. Viability was over 95% in all experiments. Cells were seeded at a density of 5 x 10^5 cells/well in 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 pIRES$_2$-EGFP, pIRES$_2$-VEGF$_{165}$/EGFP, and pIRES$_2$-VEGF$_{165}$/NT-3 vectors 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. Expression of the *VEGF$_{165}$* and *NT-3* genes was analyzed by reverse transcription (RT)-PCR.

To detect the *VEGF$_{165}$* and *NT-3* mRNA expression levels in HEK293 cells that had been either mock-infected or infected with the pIRES$_2$-EGFP, pIRES$_2$-VEGF$_{165}$/EGFP, and pIRES$_2$-VEGF$_{165}$/NT-3 vectors, *VEGF$_{165}$* and *NT-3* expression was assessed after 3 days using RT-PCR. Total RNA and reverse transcription were conducted as described above. PCR amplification of the sequences from harvested cDNAs was conducted used the following primers: *VEGF$_{165}$* primer 1 (forward), 5'-CGGAATTCATGAACTTTCTGCTGTCTTGGGTGC-3' and primer 2 (reverse), 5'-CGGGATCCTCACCGCCTCGGCTTGTCA-3'; *NT-3* primer 3 (forward), 5'-AACCATGTCCATCTTGTTTTATGTGATATTTC-3' and primer 4 (reverse), 5'-GG CCGCTCATGTTCTTCCGATTT-3'; *β-actin* primer 5 (forward), 5'-AGCGGGAAATCGTGCG TGAC-3' and primer 6 (reverse), 5'-CAAGAAAGGGTGTAACGCAACTA-3'.

After 3 days of cell culture, a standard Western blotting protocol was used to detect VEGF$_{165}$ and NT-3 protein expression in HEK293 cells that had been either mock-infected or infected with the pIRES$_2$-EGFP, pIRES$_2$-VEGF$_{165}$/EGFP and pIRES$_2$-VEGF$_{165}$/NT-3 vectors. We collected the culture supernatants of infected HEK293 cells for Western blotting using an anti-VEGF$_{165}$ and anti-NT-3 antibody. Next, 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 difluoride membranes. The membranes were incubated in a 1:500 dilution of polyclonal rabbit anti-VEGF$_{165}$ antibody (Santa Cruz Biotechnology; Santa Cruz, CA, USA) or 1:500 dilution of polyclonal rabbit anti-NT-3 antibody (Santa Cruz Biotechnology); polyclonal rabbit anti-β-actin (1:500, Santa-Cruz) was used as a loading control. After washing with Tris-buffered saline 3 times (10 min each wash) followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000) at room temperature for 2 h, membranes were washed with Tris-buffered saline (3 times for 15 min each wash), and exposed to X-ray film to observe the results. The signal was quantified using the Gel-Pro analyzer software.

Statistical analysis

The results are reported as means ± standard deviation, which were recorded from experiments. Significant differences were determined by one-way analysis of variance.

RESULTS

Amplification of *VEGF$_{165}$* and *NT-3* genes

The *NT-3* gene was obtained from the genomic DNA of human peripheral blood MNCs by PCR. The size of the *VEGF$_{165}$* gene was 576 bp, and the *VEGF$_{165}$* gene was obtained from the pIRES$_2$-VEGF$_{165}$-EGFP plasmid by PCR. The size of the *NT-3* gene was 774 bp (Figure 2A).

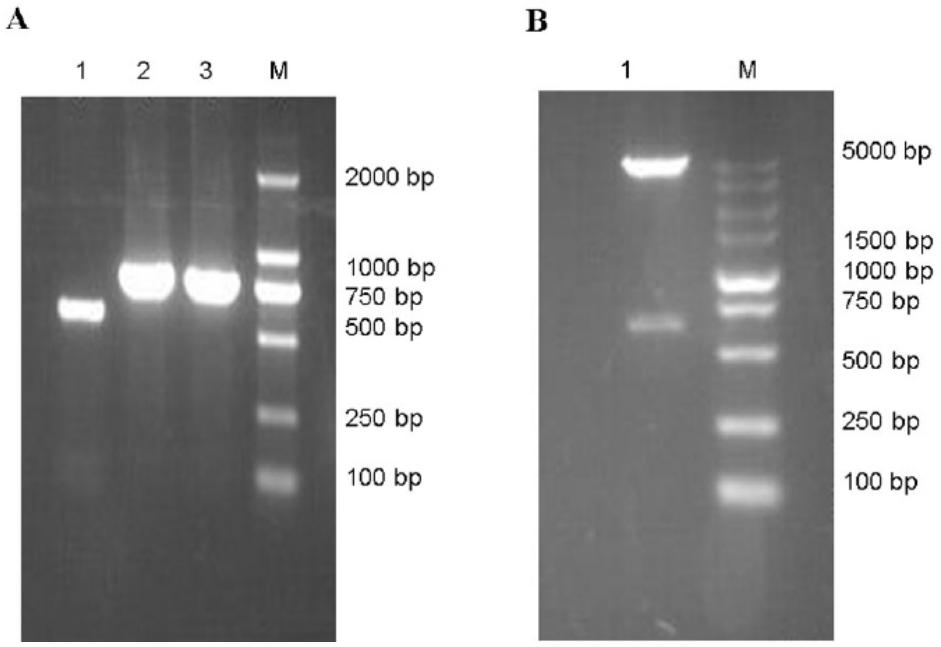

Figure 2. A. Amplification of *VEGF$_{165}$* and *NT-3*. *Lane M* = marker; *lane 1 = VEGF$_{165}$* gene; *lane 2 = NT-3* (forward-long/reverse-long primer pairs) gene; *lane 3 = NT-3* (forward-short/reverse-short primer pairs) gene. **B.** Identification of plasmid pIRES$_2$-VEGF$_{165}$-EGFP. *Lane M* = marker; *lane 1* = pIRES$_2$-VEGF$_{165}$-EGFP cut by *Eco*RI and *Bam*HI double digestion.

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

The plasmid pIRES$_2$-VEGF$_{165}$-EGFP was cut by *Eco*RI and *Bam*HI double digestion. A gene fragment of 576 bp was obtained, which correlated with the size of the *VEGF$_{165}$* gene (Figure 2B).

Identification of plasmid pIRES$_2$-VEGF$_{165}$-NT-3

The plasmid pIRES$_2$-VEGF$_{165}$-NT-3 was cut by *Eco*RI and *Bam*HI double digestion. A fragment of approximately 576 bp was obtained. This indicated that the *VEGF$_{165}$* gene had been inserted into the plasmid pIRES$_2$-VEGF$_{165}$-NT-3. The sequence of the plasmid pIRES$_2$-VEGF$_{165}$-NT-3 was in accordance with gene sequence in GenBank (Figure 3).

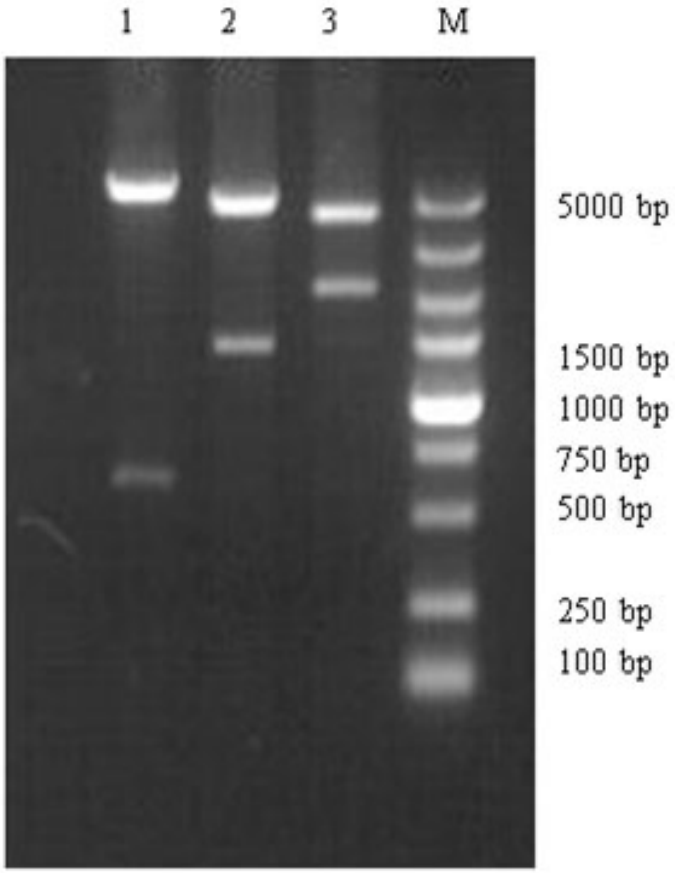

Figure 3. Identification of plasmid pIRES$_2$-VEGF$_{165}$-NT-3. *Lane M* = marker; *lane 1* = pIRES$_2$-VEGF$_{165}$-NT-3 cut by *Eco*RI and *Bam*HI; *lane 2* = pIRES$_2$-VEGF$_{165}$-NT-3 cut by *Not*I and *Bam*HI; *lane 3* = pIRES$_2$- VEGF$_{165}$-NT-3 cut by *Eco*RI and *Not*I.

RT-PCR analysis of *VEGF$_{165}$* and *NT-3* expression

To observe mRNA expression by pIRES$_2$-EGFP-, pIRES$_2$-VEGF$_{165}$/EGFP-, and pIRES$_2$-VEGF$_{165}$/NT-3-transduced HEK293 cells, we evaluated the expression of *VEGF$_{165}$* and *NT-3* using RT-PCR analysis. RT-PCR was performed using *VEGF$_{165}$*-specific primers and the *β-actin* sequence as an internal standard. GFP expression was monitored in pIRES$_2$-EGFP- and pIRES$_2$-VEGF$_{165}$/EGFP-transduced HEK293 cells using inverted fluorescence microscopy. Expression of *VEGF$_{165}$* mRNA was higher in both pIRES$_2$-VEGF$_{165}$/EGFP- and pIRES$_2$-VEGF$_{165}$/NT-3-transduced HEK293 cells than in pIRES$_2$-EGFP-transduced HEK293 cells and negative controls (Figure 4A and B). Expression of *NT-3* mRNA was higher in pIRES$_2$-VEGF$_{165}$/NT-3-transduced HEK293 cells than in the other cells (Figure 4C and D). These results demonstrate that *VEGF$_{165}$* and *NT-3* had been successfully introduced into HEK293 cells by pIRES$_2$-VEGF$_{165}$/EGFP and pIRES$_2$-VEGF$_{165}$/NT-3. After the HEK293 cells were

transduced with pIRES$_2$-VEGF$_{165}$/EGFP, the cells were passaged continually and the mean percentage of expression of GFP was monitored under fluorescence microscopy. There was no decrease in GFP fluorescence, illustrating that the transgenes were continually expressed in the transduced cells.

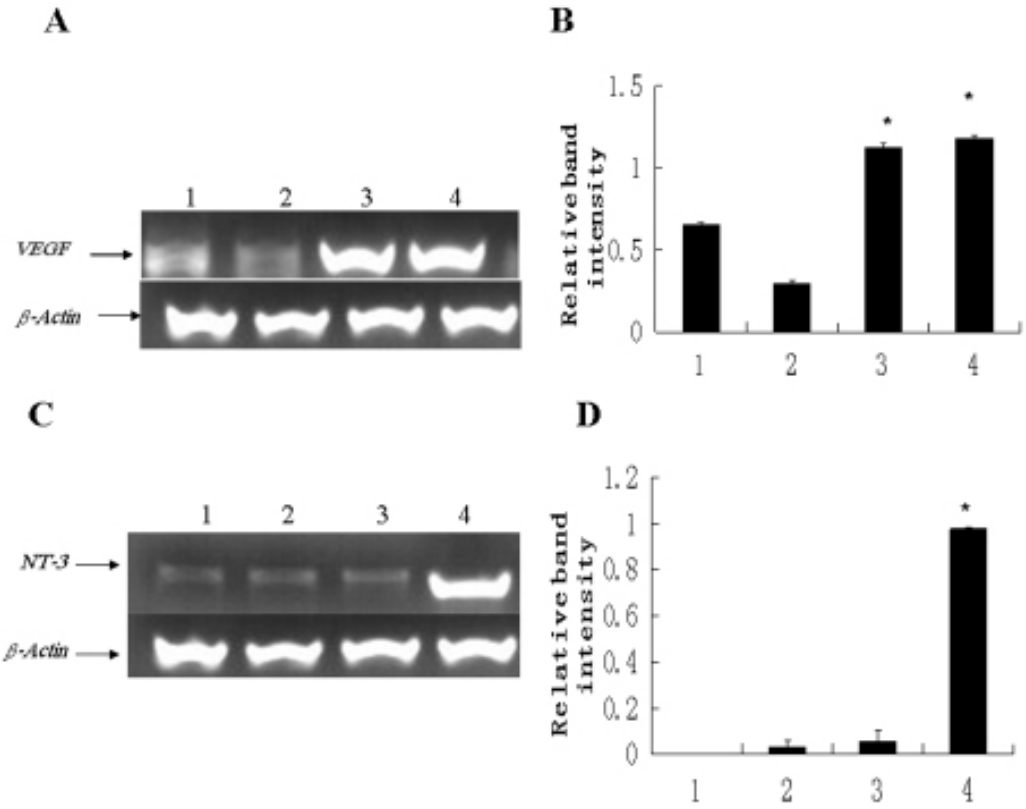

Figure 4. A. B. RT-PCR analysis of *VEGF$_{165}$* expression. *VEGF$_{165}$* = 576 bp; *β-actin* = 551-bp. **A.** *Lane 1* = non-transduced HEK293 cells; *lane 2* = GFP-transduced HEK293 cells; *lane 3* = VEGF$_{165}$-transduced HEK293 cells; *lane 4* = VEGF$_{165}$/NT-3-transduced HEK293 cells. **B.** mRNA expression level. The corresponding optical density ratio of *VEGF$_{165}$/β-actin*. Data are reported as means ± standard deviation (N = 3). Comparison of non-transduced cells and cells transduced with pIRES2-EGFP, *P < 0.01. **C. D.** RT-PCR analysis of *NT-3* expression. *NT-3* = 774 bp; *β-actin* = 551 bp. **C.** *Lane 1* = non-transduced HEK293 cells; *lane 2* = GFP-transduced HEK293 cells; *lane 3* = VEGF$_{165}$-transduced HEK293 cells; *lane 4* = VEGF$_{165}$/NT-3-transduced HEK293 cells. **D.** mRNA expression level. Corresponding optical density ratio of *NT-3/β-actin*. Data are reported as means ± standard deviation (N = 3). Comparison of non-transduced HEK293 cells and those transduced with pIRES$_2$-EGFP. *P < 0.01.

Western blot analysis of VEGF$_{165}$ and NT-3 expression in HEK293 cells

To determine protein expression by pIRES$_2$-EGFP-, pIRES$_2$-VEGF$_{165}$/EGFP-, and pIRES$_2$-VEGF$_{165}$/NT-3-transduced HEK293 cells, we evaluated the expression of VEGF$_{165}$ and NT-3 by Western blot analysis. After 72 h of transduction, we collected the culture super-

natants of infected HEK293 cells without serum and subjected the cells to Western blot analysis using anti-$VEGF_{165}$ and anti-NT-3 antibodies. β-actin was used as an internal standard. The results suggested that exogenous $VEGF_{165}$ protein was strongly expressed in $pIRES_2$-$VEGF_{165}$/EGFP- and $pIRES_2$-$VEGF_{165}$/NT-3-transduced HEK293 cells, but the $pIRES_2$-EGFP-transduced HEK293 cells and non-transduced cell expression of endogenous $VEGF_{165}$ was very low (Figure 5A and B). Similarly, Western blot analysis also revealed that exogenous NT-3 protein was strongly expressed in $pIRES_2$-$VEGF_{165}$/NT-3-transduced HEK293 cells, and that the $pIRES_2$-EGFP- and $pIRES_2$-$VEGF_{165}$/EGFP-transduced HEK293 cells expressed of endogenous NT-3 at very low levels (Figure 5C and D). These results also demonstrated that $VEGF_{165}$ and *NT-3* were introduced successfully into HEK293 cells through the $pIRES_2$-$VEGF_{165}$/NT-3 vector.

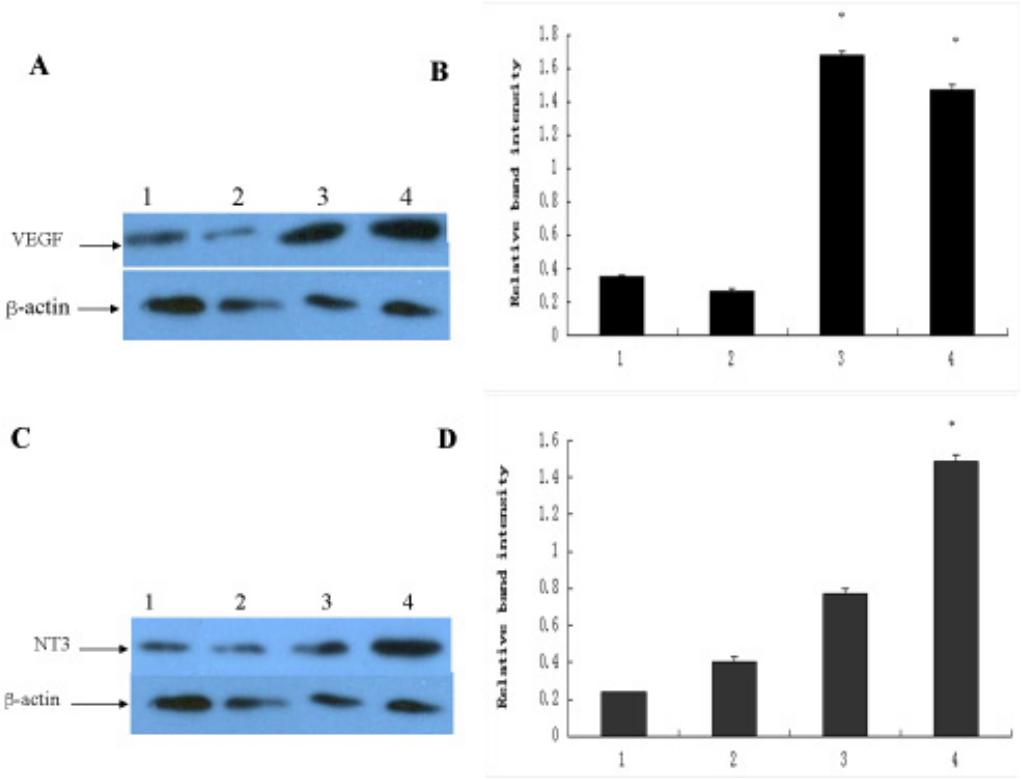

Figure 5. A. B. Western blot analysis of $VEGF_{165}$ expression. $VEGF_{165}$ = 38.2 kDa; β-actin = 42 kDa. **A.** *Lane 1* = non-transduced HEK293 cells; *lane 2* = GFP-transduced HEK293 cells; *lane 3* = $VEGF_{165}$-transduced HEK293 cells; *lane 4* = $VEGF_{165}$/NT-3-transduced HEK293 cells. **B.** Corresponding optical density ratio of $VEGF_{165}$/β-actin. Data are reported as means ± standard deviation (N = 3). Compared with non-transduced cells or cells transduced with $pIRES_2$-EGFP. *P < 0.01. **C. D.** Western blot analysis of NT-3 expression. NT-3 = 27 kDa; β-actin = 42 kDa. **C.** *Lane 1* = non-transduced HEK293 cells; *lane 2* = GFP-transduced HEK293 cells; *lane 3* = $VEGF_{165}$-transduced HEK293 cells; *lane 4* = $VEGF_{165}$/NT-3-transduced HEK293 cells. **D.** The protein expression level. Corresponding optical density ratio of NT-3/β-actin. Data are reported as means ± standard deviation (N = 3). Comparison of non-transduced cells or cells transduced with $pIRES_2$-EGFP, $pIRES_2$-$VEGF_{165}$/EGFP. *P < 0.05.

DISCUSSION

To improve cloning efficiency and simplify the cloning procedure, we developed a twin PCR method to insert DNA with single-stranded overhangs at the ends complementary to a restriction enzyme-digested vector (Xie and Xie, 2011). As shown in Figure 1, PCR products were reassembled via complementary pairing after heat-denaturing and re-annealing. Four types of molecules of equal proportions were created, 2 of which were blunt-ended and another 2 that were sticky-ended. Half of the sticky-ended products were suitable for ligation. Using our twin PCR strategy, hybridized PCR products flanked with various types of sticky ends that are compatible with nearly all restriction enzymes could be generated through primer design and PCR amplification. Because no restriction enzyme digestion was required for the PCR products, twin PCR overcomes the limitation of restriction enzyme sensitivity, 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 a larger number of restriction enzymes sites.

In this study, *NT-3* was directly inserted into pIRES$_2$-VEGF$_{165}$-EGFP at the *Bst*XI and *Not*I sites. These efficient and economical enzymes, such as *Bst*XI and *Not*I, can be used for vector digestion, reducing the number of enzymes required for cloning. In addition, our simple procedure can be completed in 8 h, saving substantial amounts of time, financial resources, and labor. Furthermore, inserts are amplified using proofreading DNA polymerase, which provides a faithful guarantee of the PCR products. These advantages make our method universally advantageous 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, chrondogenic, and adipogenic lineages in response to stimulation by multiple environmental factors (Baksh et al., 2004). Various cytokines and transcription factors play an important role in this procedure.

VEGF, originally identified based on 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 prevents the development of diabetic neuropathy in rodents and rabbits (Yoon et al., 2009). VEGF was previously shown to have 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 are increased in Schwann cells of rats with diabetic neuropathy, perhaps in response to nerve hypoxia (Chattopadhyay et al., 2005). Systemic expression of VEGF achieved through the intramuscular gene transfer of naked DNA encoding VEGF has been shown to restore 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 for VEGF$_{165}$ was injected into the muscles of patients with leg ischemia, resulting in significant clinical improvement in neuropathic symptoms, nerve conduction measures, and vibration threshold (Malik and Veves, 2007).

To date, NT-3 is the only neurotrophic factor identified to promote the growth of corticospinal axons after spinal cord injury (Bradbury et al., 1999). *NT-3* gene delivery promoted corticospinal axon growth distal to the lesion site, perhaps as a result of a serendipitous event: corticospinal axons did not penetrate the NT-3-secreting cell grafts. Confirming the finding

that NT-3 supports corticospinal axon growth, Blesch et al. (1999) used an *ex vivo* gene delivery approach to deliver the cytokine growth factor leukemia inhibitory factor (*LIF*) to the spinal cord (Jones et al., 2001). *LIF* gene delivery upregulated NT-3 expression in the spinal cord, and corticospinal axonal growth was significantly increased in grey matter at the lesion site. This study also highlighted that delivery of 1 growth factor to the CNS may augment the production of other growth factors, thereby sustaining neuronal growth or survival. In another study, continuous infusion of *NT-3* into the dorsal spinal cord was reported to promote the growth of sensory axons from a peripheral nerve graft into the dorsal column white matter (Blesch et al., 2012). A variety of axonal populations of the spinal cord respond to neurotrophic factors (Brock et al., 2010). Determining the specific patterns of sensitivity will allow the design of rational strategies for promoting more extensive axonal growth in the context of injury and degeneration.

In this study, the $VEGF_{165}$ and *NT-3* genes were inserted into a bicistronic eukaryotic expression vector. The plasmid pIRES$_2$-VEGF$_{165}$-NT-3 was successfully constructed. The plasmid was then transfected into MSCs in the next experiment and the $VEGF_{165}$ and *NT-3* genes were expressed in the cells. Combination gene therapy with both genes may have more significant effects on neuron regeneration than the single genes alone.

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