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Construction and identification of pIRES$_2$-LIF-NT-3 bicistronic eukaryotic expression vector

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ABSTRACT. We used a simple and efficient method to construct a bicistronic eukaryotic expression vector pIRES$_2$-LIF-NT-3. The leukemia inhibitory factor (LIF) and neurotrophin-3 (NT-3) genes were obtained from the genomic DNA of human peripheral blood mononuclear cells by polymerase chain reaction. The LIF cDNA fragment was inserted into the multiple cloning sites of a vector containing internal ribosome entry site and enhanced green fluorescent protein (EGFP) (pIRES$_2$-EGFP) to generate the bicistronic eukaryotic expression plasmid pIRES$_2$-LIF-EGFP. Next, the NT-3 cDNA fragment was cloned into pIRES$_2$-LIF-EGFP in place of EGFP to create the plasmid pIRES$_2$-LIF-NT-3. pIRES$_2$-LIF-NT-3 was transfected into HEK293 cells and reverse transcription-polymerase chain reaction and Western blotting were used to test the co-expression of double genes. LIF and NT-3 genes were cloned and the DNA was sequenced. Sequencing analysis revealed that LIF and NT-3 were consistent with the sequence recorded in GenBank. Restriction analysis indicated that the LIF and NT-3 genes were inserted correctly into the expression

vector pIRES$_2$-EGFP. Following transfection of pIRES$_2$-LIF-NT-3 into HEK293 cells, the double gene was expressed at the mRNA and protein levels. The LIF and NT-3 coexpression plasmid is a novel expression system that will enable further study of the functions of the LIF and NT-3 genes.

Key words: Bicistronic eukaryotic expression vector; Neurotrophin-3; Leukemia inhibitory factor; Internal ribosome entry site

INTRODUCTION

Injury to the adult mammalian spinal cord results in extensive axonal degeneration, variable amounts of neuronal loss, and often severe functional deficits (Ruff et al., 2012). Restoration of controlled function depends on the regeneration of these axons at the injury site and the formation of functional synaptic connections (Wang and Sun, 2011). One strategy for promoting axonal regeneration after spinal cord injury is implanting stem cells into spinal cord injury sites to support and guide axonal growth (Straley et al., 2010). Further, recent experiments have shown that neurotrophic factors can promote axonal growth, and, when combined with stem cell grafts, amplify axonal extension after injury (Ziv-Polat et al., 2012). Continued preclinical development of these approaches for neural repair may generate strategies that can be tested for treating human injury.

Mesenchymal stem cells (MSCs) are thought to be a good source of stem cells because of their tremendous self-renewal and multi-lineage differentiation potential (Huang et al., 2010). Curative effects have been observed in animal experiments involving the use of MSCs to treat ischemic brain injury (Maksymowicz et al., 2012). Human bone marrow cells have been extensively used to treat hematologic diseases (Senn et al., 2012). Moreover, non-hematopoietic stem cells, such as MSCs, can differentiate into mature osteocytes, chondrocytes, and adipocytes (Liu et al., 2009). Previous studies have found that bone marrow cells (BMCs) differentiate into mature neurons or glial cells when induced under experimental conditions (Ribeiro et al., 2011).

These findings indicate that BMCs can be applied therapeutically in patients with neurological diseases, which would overcome ethical problems related to the use of embryonic stem cells.

Enhancing the proliferation and orientation differentiation ability of stem cells by gene modification has received increased attention in tissue engineering fields. Leukemia inhibitory factor (Hawryluk et al., 2012), neurotrophin-3 (Schnell et al., 2011), nerve growth factor, and brain-derived neurotrophic factor or the derived scaffolds or vectors constructed based on these factors have been widely examined in experiments and gradually applied in clinical use. In this study, we constructed the bicistronic eukaryotic expression vector pIRES$_2$-LIF-NT-3 using a simple and efficient method, which will enable further studies of LIF and NT-3 gene function.

MATERIAL AND METHODS

Materials

pIRES$_2$-EGFP (Beijing Tian ENZE Gene Technology Co., Ltd, Beijing, China), hu-

man peripheral blood mononuclear cells from healthy donors, Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA), T4 DNA ligase, 2X Prime STAR Max DNA Polymerase, the *Bam*HI, *Bst*XI, *Eco*RI, and *Not*I restriction enzymes, the total RNA extraction kit, reverse transcription kit, high-purity gel extraction kit, and DNA marker were from Takara Biotechnology (Shiga, Japan).

Primer design

The LIF (NM_002309.4) and NT-3 (GenBank No. NM_002527) gene sequences in GenBank served as templates and were used to design the primers.

Overlap-polymerase chain reaction (PCR), an efficient and rapid method, was used to clone the human LIF gene coding sequence (CDS) from genomic DNA. The procedure included 7 primers and 3-step PCRs. The CDS of human LIF gene fragment included 2 main exons (exon1 + exon2). Two primer pairs were designed to amplify LIF-22, which contained exon1 (LIF-2). The forward primer sequence was 5'-TCTCCCATGCGGCCATTGTTG-3' and the reverse primer sequence was 5'-CCTCCCTGCCATCTCCTGTCAGTATC-3'; 2 other primer pairs were designed to amplify exon1 (LIF-2) from the LIF-22 DNA fragment. The forward primer was 5'-GCCCTCTGAAGTGCAGCCCATAA-3' and the reverse primer was 5'-CTGGGCTGTGTAATAGAGAATAAAGAGGG-3'. Two other primer pairs were designed to amplify exon2 (LIF-3). The forward primer was 5'-CTTTATTCTCTATTACACAGCCCAGG-3' and the reverse primer was 5'-CGGGATCCCTAGAAGGCCTGGGCCAACACG-3'. The *Bam*HI restriction enzyme cut site was added to this primer because it was the outermost primer downstream of the gene. The outermost upstream primer was 5'-CGGAATTCATGAAGGTCTTGGCGGCAGG-3' The *Eco*RI restriction enzyme cut site was added to this primer. The length of the CDS was 609 base pairs (bp).

Two primer pairs were designed for amplification of 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, similarly to the reverse-long primer (5'-GGCCGCTCATGTTCTTCCGATTT-3') compared to the reverse-short primer (5'-GCTCATGTTCTTCCGATTTTTCTCGAC-3'). The underlined bases introduced a *Bst*XI site in the forward primer and *Not*I site in the reverse primer, which were used to create the *Bst*XI and *Not*I sticky ends, and the length of the amplified fragment was 774 bp.

Construction of pIRES$_2$-LIF-EGFP

The genomic DNA of human peripheral blood MNCs served as the template and the primer for LIF was used to amplify the LIF gene. In this study, overlap-PCR, an efficient and rapid method, was used to clone the human LIF gene CDS from genomic DNA. The procedure included 7 primers and 3-step PCR. The human LIF gene consists of 2 main exons and the CDS contains 609 bp. In the first step, 3 PCRs were performed to generate extended exon1 (LIF-2) from the LIF-22 and exon2 (LIF-3) that contained overlapping nucleotides, which were used as the templates for the second ligation PCR. Two primer pairs were designed to amplify LIF-22, which contains the exon1 (LIF-2). Two other primer pairs were designed to amplify the exon1 (LIF-2) from LIF-22 DNA fragment and another 2 primer pairs were de-

signed to amplify the exon2 (LIF-3). Second, exon1 (LIF-2) and exon2 (LIF-3) were spliced together. Finally, the 2 exons (LIF-2 and LIF-3) were linked together with outermost primers to the templates from the second step. As an efficient and rapid method, overlap-PCR is feasible for gene cloning from genomic DNA.

A 20-μL PCR reaction system was used, which included 10 ng 0.5 μL genomic DNA, 10 μL 2X Prime STAR Max DNA Polymerase, 7.5 μL RNase-Free Water, and 2 μL each upstream primer and downstream primer. The reaction conditions were as follows: 95°C for 5 min, 98°C for 10 s, 55°C for 5 s, and 72°C for 5 s for 30 cycles, and a final 72°C extension for 5 min. PCR products were purified using the PCR Purification Kit, and the PCR product and plasmid pIRES$_2$-EGFP were cut by using *Eco*RI 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 under the following conditions for a 20-μL reaction: 2 μL pIRES2-EGFP, 8 μL cDNA (LIF), and 0.2 μL T4 DNA ligase at 22°C for 30 min. The DNA fragment was transformed into *Escherichia coli* DH5α and placed on an LB plate (kan^r) at 37°C in an incubator for 16 h. Monoclonal colonies were picked up and shaken for 12-16 h at 37°C at a speed of 225 revolutions/min. The plasmid was extracted and identified using *Eco*RI and *Bam*HI double enzyme digestion. The recombinant plasmid pIRES$_2$-LIF-EGFP was obtained.

Construction of pIRES$_2$/LIF-NT-3

A simple and efficient method was developed for directional cloning of PCR products without a restriction enzyme digestion step 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 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 the restriction enzymes.

PCR amplification of NT-3

Two parallel PCRs were set up using either forward-long/reverse-long or forward-short/reverse-short primer pairs, with the genomic DNA of human peripheral blood MNCs as templates. Both amplifications 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 2X 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 the Nano Drop2000UV-Vis spectrophotometer (Nanodrop, Inc., Wilmington, DE, USA) (Figure 1).

Cloning of PCR products

pIRES$_2$-LIF-EGFP was digested with *Bst*XI and *Not*I, followed by phenol:chloroform extraction and ethanol precipitation. Additionally, each pooled NT-3 PCR product was amplified with 2X Prime STAR Max DNA Polymerase mixed, denatured at 94°C for 4 min, and re-annealed at 65°C for 2 min. For the ligation reaction, the annealed NT-3 and linearized

pIRES$_2$-LIF-EGFP (molar ratio of 4:1) were incubated with T4 DNA ligase at 16°C for 3 h. For TA cloning, the 2 PCR products amplified by EXTaq polymerase were mixed in equimolar ratios for ligation into pMD18-T according to manufacturer instructions. The 2 ligation products were used to transform *E. coli* DH5< $CaCl_2$ competent cells following a standard protocol. Transformants were screened by *Bst*XI and *Not*I digestion following alkaline lysis plasmid preparation, and the sequences of the recombinants were confirmed by DNA sequencing.

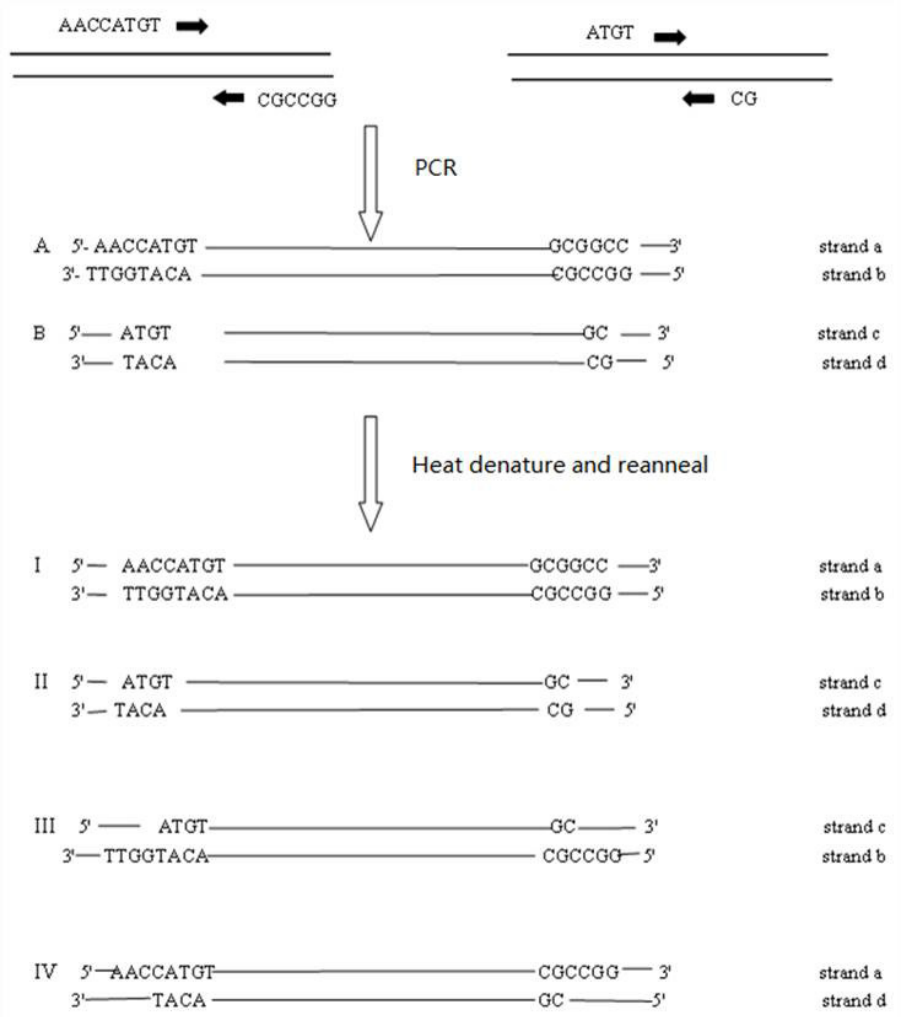

Figure 1. Outline of twin PCR. Two parallel PCRs were set up using either forward-long/reverse-long (PCR products **A**) or forward-short/reverse-short primer pairs (PCR products **B**), with the genomic DNA of human peripheral blood mononuclear cells as templates. Equimolar amounts of PCR products **A** 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 these, I and II, were blunt-ended and the other 2 were sticky-ended. Molecule III possess a 5' *Bst*XI sticky end and a 3' *Not*I sticky end, allowing the fragment to be cloned directionally into pIRES$_2$-LIF-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 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 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$_2$-LIF/EGFP, or pIRES$_2$-LIF/NT-3 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. LIF and NT-3 gene expression was analyzed by reverse transcription (RT)-PCR.

To detect the LIF and NT-3 mRNA expression levels in HEK293 cells that had been either mock-infected or infected with the pIRES$_2$-EGFP, pIRES$_2$-LIF/EGFP, and pIRES$_2$-LIF/NT-3 vector, after 3 days, LIF and NT-3 expression was primarily assessed by means of RT-PCR. The methods used for extracting total RNA and reverse transcription were as described above. PCR amplification of these sequences from harvested cDNAs used these primers: LIF, primer 1 (forward), 5'-CGGAATTCATGAAGGTCTTGGCGGCAGG-3' and primer 2 (reverse), 5'-CGGGATCCCTAGAAGGCCTGGGCCAACACG-3'. The NT-3 primers were primer 3 (forward), 5'-AACCATGTCCATCTTGTTTTATGTGATATTTC-3', and primer 4 (reverse), 5'-GGCCGCTCATGTTCTTCCGATTT-3'. The β-actin primers were primer 5 (forward), 5'-AGCGGGAAATCGTGCGTGAC-3', and primer 6 (reverse), 5'-CAAGAAAGGGTGTAACGCAACTA-3'.

A standard Western blotting protocol was used to detect LIF and NT-3 protein expression in HEK293 cells, which had been either mock-infected or infected with the pIRES$_2$-EGFP, pIRES$_2$-LIF/EGFP, and pIRES$_2$-LIF/NT-3 vector. Three days later, we collected the culture supernatants of infected HEK293 cells without serum, which were processed for Western blotting using an anti-LIF and anti-NT-3 antibody. Next, 20 μg protein was extracted from transduced HEK293 cells and 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-LIF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 1:500 dilution of polyclonal rabbit anti-NT-3 antibody (Santa Cruz Biotechnology), and 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 time), the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit lgG (1:5000) at room temperature for 2 h. Tris-buffered saline was used to wash the membranes 3 times for 15 min each time, and exposed to X-ray film to detect the results. The amount of signal was quantified using the Gel-Pro analyzer software.

Statistical analysis

The results are reported as means ± standard deviation and were recorded from the 5 experiments. The significance of differences was determined by 1-way analysis of variance.

RESULTS

Amplification of LIF and NT-3 genes

LIF-22 and LIF-3 (exon 2) were obtained from the genomic DNA of human peripheral blood MNCs (Figure 2A). LIF-2 (exon1) was obtained from LIF-22 (Figure 2B). Exon1 (LIF-2) and exon2 (LIF-3) were spliced together by overlap-PCR (Figure 2C). The size of the LIF gene was 609 bp. NT-3 genes were obtained from the genomic DNA of human peripheral blood MNCs by PCR. The size of the NT-3 gene was 774 bp (Figure 2D).

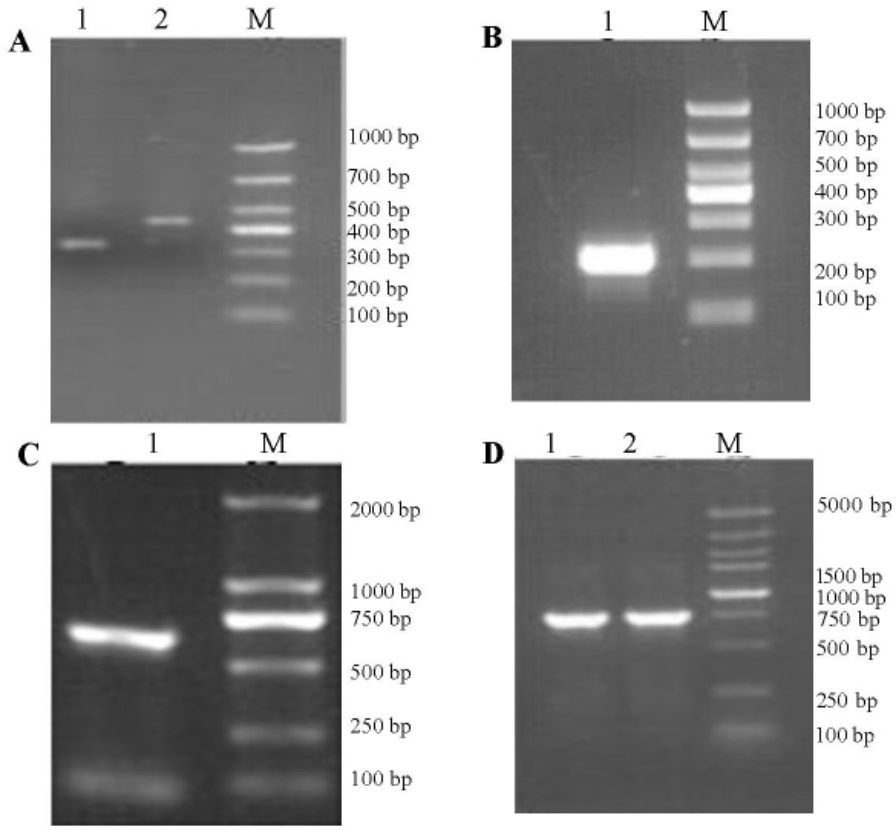

Figure 2. Amplification of LIF and NT-3. **A.** Amplification of LIF-22 and LIF-3 (exon 2). *Lane M* = marker; *lane 1* = LIF-22 fragment; *lane 2* = LIF-3 (exon2) gene. **B.** Amplification of LIF-2 (exon 1) from LIF-22. *Lane M* = marker; *lane 1* = LIF-2 (exon 1) gene. **C.** Amplification of LIF. *Lane M* = marker; *lane 1* = LIF (exon 1 + exon2) gene. **D.** Amplification of NT-3. *Lane M* = marker; *lane 1* = NT-3 gene (forward-long/reverse-long primer pairs); *lane 2* = NT-3 gene (forward-short /reverse-short primer pairs).

Identification of plasmid pIRES$_2$-LIF-EGFP

The plasmid pIRES$_2$-LIF-EGFP was cut by *Eco*RI and *Bam*HI double digestion. A gene fragment of 609 bp was obtained, which agreed with the expected size of the LIF gene (Figure 3).

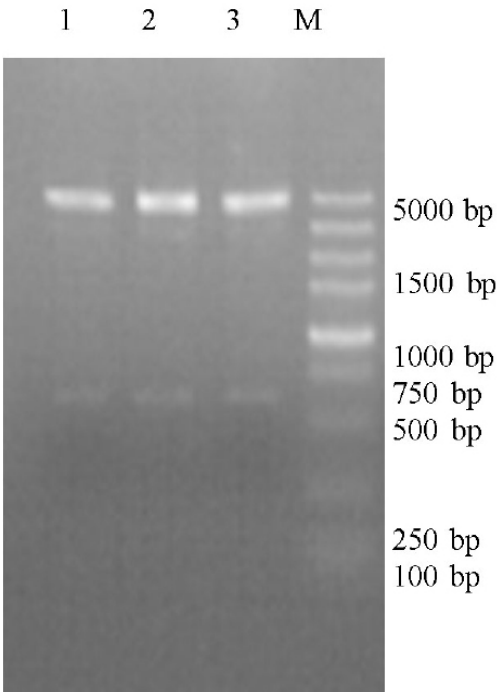

Figure 3. Identification of plasmid pIRES2-LIF-EGFP. *Lane M* = marker; *lanes 1-3* = pIRES2-LIF-EGFP cut by *Eco*RI and *Bam*HI double digestion.

Identification of plasmid pIRES$_2$-LIF-NT-3

The plasmid pIRES$_2$-LIF-NT-3 was cut by *Eco*RI and *Bam*HI double digestion. A fragment approximately 609 bp was obtained, indicating that the LIF gene had been inserted into the plasmid pIRES-LIF-NT-3. The sequence of the plasmid pIRES-LIF-NT-3 was in accordance with gene sequence in GenBank (Figure 4).

RT-PCR analysis the expression of LIF and NT-3

To determine the mRNA expression by pIRES$_2$-EGFP, pIRES$_2$-LIF/EGFP, and pIRES$_2$-LIF/NT-3 in transduced HEK293 cells, we evaluated the expression of LIF and NT-3 by RT-PCR analysis. RT-PCR was performed using LIF-specific primers and the β-actin sequence as an internal standard. GFP expression was monitored in pIRES$_2$-EGFP and pIRES$_2$-LIF/EGFP transduced HEK293 cells by inverted fluorescence microscopy. The expression of LIF mRNA was higher in both pIRES$_2$-LIF/EGFP and pIRES$_2$-LIF/NT-3 transduced HEK293 cells compared to in the pIRES$_2$-EGFP transduced HEK293 cells or in negative controls (Figure 5A and B). As shown above, the expression of NT-3 mRNA was higher in pIRES$_2$-LIF/NT-3-transduced HEK293 cells than in the other 3 transduced cells (Figure 5C and D). These results demonstrated that LIF and NT-3 had been successfully introduced into HEK293 cells by pIRES$_2$-LIF/EGFP and pIRES$_2$-LIF/NT-3. After the HEK293 cells were transduced with

pIRES$_2$-LIF/EGFP, we passaged the cells continually and then monitored the mean percentage of GFP expression under fluorescence microscopy. There was no decrease in GFP fluorescence, illustrating the maintenance of transgenic expression in the transduced cells.

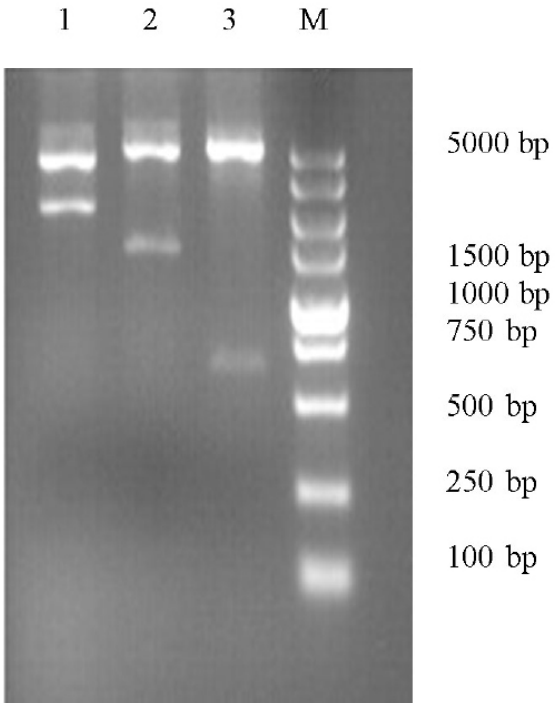

Figure 4. Identification of plasmid pIRES2-LIF-NT-3. *Lanes M* = marker; *lane 1* = pIRES2-LIF-NT-3 cut by *Eco*RI and *Not*I; *lane 2* = pIRES2-LIF-NT-3 cut by *Bam*HI and *Not*I; *lane 3* = pIRES2-LIF-NT-3 cut by *Bam*HI and *Eco*RI.

Western blot analysis of LIF and NT-3 expression in HEK293 cells

To observe protein expression for pIRES$_2$-EGFP, pIRES$_2$-LIF/EGFP, and pIRES$_2$-LIF/NT-3 transduced HEK293 cells, we evaluated the expression of LIF and NT-3 by Western blotting. After 72 h of transduction, we collected the culture supernatants of infected HEK293 cells without serum, which were processed for Western blotting using anti-LIF and anti-NT-3 antibodies. β-actin was used as an internal standard, and the results suggested that exogenous LIF protein was strongly expressed in pIRES$_2$-LIF/EGFP and pIRES$_2$-LIF/NT-3 transduced HEK293 cells, but the pIRES$_2$-EGFP transduced HEK293 cells and non-transduced expression of endogenous LIF was very low (Figure 6A and B). As shown above, Western blot analysis also revealed that exogenous NT-3 protein was strongly expressed in pIRES$_2$-LIF/NT-3 transduced HEK293 cells, and in pIRES$_2$-EGFP and pIRES$_2$-LIF/EGFP transduced HEK293 cells, expression of endogenous NT-3 was very low (Figure 6C and D). These results also demonstrated that LIF and NT-3 had been successfully introduced into HEK293 cells by pIRES$_2$-LIF/NT-3.

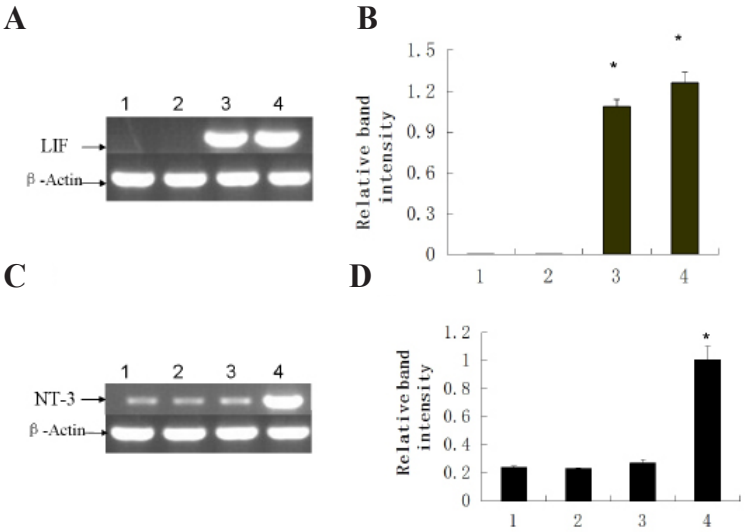

Figure 5. A. B. RT-PCR analysis of LIF expression. LIF = 609 bp; β-actin = 551 bp. **A.** *Lane 1* = NO transduced HEK293 cells; *lane 2* = GFP transduced HEK293 cells; *lane 3* = LIF transduced HEK293 cells; *lane 4* = LIF/NT-3 transduced HEK293 cells. **B.** mRNA expression level. OD ratio of LIF/β-actin. Data are reported as means ± SD (N = 3). Compared with NO transduced or transduced by 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* = NO transduced HEK293 cells; *lane 2* = GFP transduced HEK293 cells; *lane 3* = LIF transduced HEK293 cells; *lane 4* = LIF/NT-3 transduced HEK293 cells. **D.** mRNA expression level. OD ratio of NT-3/β-actin. Data are reported as means ± SD (N = 3). Compared with NO transduced HEK293 cells or transduced by pIRES2-EGFP. *P < 0.01.

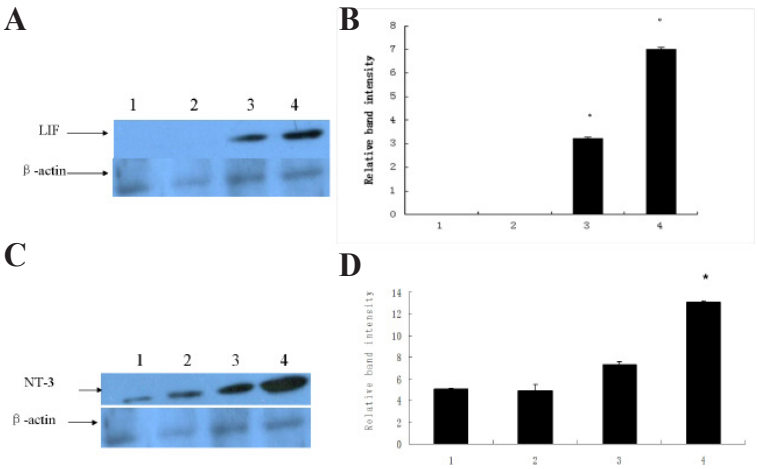

Figure 6. A. B. Western blot analysis of LIF expression. LIF (19.7 kDa), β-actin (42 kDa). **A.** *Lane 1* = non-transduced HEK293 cells; *lane 2* = GFP transduced HEK293 cells; *lane 3* = LIF transduced HEK293 cells; *lane 4* = LIF/NT-3 transduced HEK293 cells. **B.** Correspondence OD ratio of LIF/β-actin. Data are reported as means ± SD (N = 3). Compared with non-transduced or transduced with pIRES2-EGFP. *P < 0.05. **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* = LIF transduced HEK293 cells; *lane 4* = LIF/NT-3 transduced HEK293 cells. **D.** Protein expression level. OD ratio of NT-3/β-actin. Data are reported as means ± SD (N = 3). Compared with non-transduced or transduced with pIRES2-EGFP, pIRES2-LIF/EGFP. *P < 0.05.

DISCUSSION

To improve the cloning efficiency and to simplify the cloning procedure, we developed twin PCR to create an insert DNA with single-stranded overhangs, which were complimentary to those in a restriction enzyme digested vector (Xie and Xie, 2011). As shown in Figure 1, the PCR products reassembled via complimentary pairing after heat-denaturing and re-annealing. Four types of molecules with equal proportions were created, 2 of which were blunt-ended and 2 that had sticky ends. Theoretically, half of the sticky-ended products are suitable for a specific ligation. Using the twin PCR strategy, hybridized PCR products flanked with all types of sticky ends that were 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 of end sensitivity of restriction enzymes, and the internal restriction sites within the amplified sequences are not limiting for the selection of cloning sites. This is particularly useful for cloning long PCR products, which may contain a larger number of restriction enzyme sites.

In this study, NT-3 was directly inserted into pIRES$_2$-LIF-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. This limits the number of restriction enzymes required for cloning. Additionally, our cloning method is simple, and the entire procedure, including PCR, enzyme digestion, ligation, and transformation, can be completed in 8 h. This saves time, cost, and labor. In addition, interested inserts can be amplified using proof-reading DNA polymerase, which ensures that the PCR product sequences will be correct. These advantages make our 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 (Baksh et al., 2004). Various cytokines and transcription factors play important roles in this procedure.

LIF, which is also known as cholinergic differentiation factor, is a multifunctional cytokine that affects neurons, as well as many other cell types (Ishibashi et al., 2009). *In vitro*, the actions of LIF on sympathetic neurons are very similar to those of ciliary neurotrophic factor. Similar to other neurotrophic factors, LIF is retrogradely transported by a subpopulation of small diameter neurons in dorsal root ganglia (Zhou et al., 1999). A large proportion of these neurons are also positive for trkA and calcitonin gene-related peptide, while the remaining is labeled by isolectin B4. This overlap between neurons accumulating LIF and those responsive to NGF is much wider than that described for cells with receptors for neurotrophin and glial cell line-derived neurotrophic factor (Terenghi, 1999). These findings and the observation that sympathetic neurons retrogradely transport both to NGF and NT-3 from peripheral target tissues are consistent with the possibility that there may be multiple growth factor controls on neuronal functions and survival (Lee et al., 2001).

To date, NT-3 is the only neurotrophic factor that has been found to promote the growth of corticospinal axons after spinal cord injury (Bradbury et al., 1999). In a more recent study, NT-3 gene delivery promoted corticospinal axon growth distal to the lesion site, perhaps because of a serendipitous event: corticospinal axons did not penetrate the NT-3-secreting cell grafts. Additionally, Blesch et al. used an *ex vivo* gene delivery approach to deliver the cytokine growth factor, LIF, to the spinal cord (Jones et al., 2001). LIF gene delivery unregu-

lated NT-3 expression in the spinal cord and corticospinal axonal growth was significantly increased in grey matter at the lesion site (Blesch et al., 1999). This study also highlighted that the 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). Identifying the specific patterns of sensitivity will allow the design of rational strategies for promoting further extensive axonal growth in case of injury and degeneration.

In this study, the LIF and NT-3 genes were inserted into a bicistronic eukaryotic expression vector. The plasmid pIRES$_2$-LIF-NT-3 was successfully constructed. The plasmid can be transfected into MSCs in future experiments and the LIF and NT-3 genes can be expressed in the cells. Thus, combined gene therapy of both genes may have more significant effects on neuron regeneration than a single gene.

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