doi: 10.13241/j.cnki.pmb.2014.31.014

Construction and Identification of pIRES2-GDNF-NT-3 Bicistronic Eukaryotic Expression Vector*

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ABSTRACT Objective: Using a simple and efficient method to construct a bi-cistronic eukaryotic expression vector pIRES2-GDNF-NT-3. **Methods:** GDNF and NT-3 genes were obtained from the genomic DNA of human peripheral blood mononuclear cells by PCR. The GDNF cDNA fragment was inserted into the multiple cloning sites of pIRES2-EGFP to generate the bi-cistronic eukaryotic expression plasmid pIRES2-GDNF-EGFP. Then NT-3 cDNA fragment was cloned into the pIRES2-GDNF-EGFP instead of EGFP creating plasmid pIRES2-GDNF-NT-3. **Results:** GDNF and NT-3 genes were cloned; the DNA sequencing analysis demonstrated that the GDNF and NT-3 were exactly consistent with the sequence recorded in GenBank. Restriction analysis indicated that GDNF and NT-3 genes were inserted expression vector pIRES2-EGFP correctly. **Conclusions:** The GDNF and NT-3 co-expression plasmid is successfully constructed. It provides a novel expression system, which makes it possible to further study on the functions of GDNF and NT-3 genes.

Key words: GDNF; NT-3; Bi-cistronic eukaryotic expression vector; Internal ribosome entry site

Chinese Library Classification (CLC): Q784; Q75 Document code: A

Article ID: 1673-6273(2014)31-6056-06

Injury to the adult mammalian spinal cord results in extensive axonal degeneration, variable amounts of neuronal loss, and often severe functional deficits [1]. Restoration of controlled function depends on regeneration of these axons through an injury site and the formation of functional synaptic connections [2]. One strategy that has emerged for promoting axonal regeneration after spinal cord injury is the implantation of stem cells into sites of spinal cord injury to support and guide axonal growth[3]. Further, more recent experiments have shown that neurotrophic factors can also promote axonal growth, and, when combined with stem cell grafts, can further amplify axonal extension after injury [4]. Continued preclinical development of these approaches to neural repair may ultimately generate strategies that could be tested in human injury.

Mesenchymal stem cells (MSCs) have been considered as a good source of stem cells due to tremendous self-renewal and multi-lineage differentiation potential [5], Some curative effects have been made in animal experiments using MSCs to treat ischemic brain injury[6]. Human bone marrow cells have a long history in the treatment of hematologic diseases [7]. Moreover, non-hematopoietic stem cells, such as mesenchymal stem cells (MSCs), can differentiate into mature osteocytes, chondrocytes, and adipocytes[8]. Some have found that bone marrow cells (BMCs) differentiate into mature neurons or glial cells when induced under experimental conditions [9]. These findings raised the possibility of applying BMCs therapeutically in patients with neurological diseases, which would also obviate ethical problems in the use of embryonic stem cells.

To enhance the proliferation and orientational differentiation ability of stem cells by gene modification is hot in tissue engineering. Glial cell line-derived neurotrophic factor, neurotrophin-3, nerve growth factor and brain derived neurophic factor or the derived scaffolds or vectors constructed based on these factors have been widely used in experiment [10] and gradually applied in the clinic. This study was to construct a bi-cistronic eukaryotic expression vector pIRES2-GDNF-NT-3 in a simple and and efficient method which enable to further study on the functions of BDNF and NT-3 genes.

1 Materials and Methods

1.1 Materials

pIRES2-EGFP (The Beijing Tian ENZE Gene Technology Co., Ltd), Human peripheral blood mononuclear cells (Healthy donors), Lipofectamine™2000(Invitrogen), T4 DNA ligase, Prime STAR Max DNA Polymerase, *Bgl*II, *Sma*I, *Not*I, *Bst*XI, restriction enzyme, Total RNA extraction kit, Reverse transcription kit, High-purity gel extraction kit, DNA marker(Takara Biotechnology (Dalian)CO., LTD.)

1.2 Design of the primers

GDNF (NM_000514.3) and NT-3 gene sequence (GenBank no.NM_002527) in Gene Bank served as the template was used to design the primers.

Overlap-PCR, an efficient and rapid method, was used to clone human glial cell derived neurotrophic factor (GDNF) gene CDS (coding sequence) from genomic DNA. The procedure included four primers and three-step PCRs. The Coding Sequence of

*Foundation item: This work was supported by the Key science and technology project of Henan Province (122101310100);
Tender Subject of Key Research Areas of Xinxiang Medical University in 2011, (ZD2011-16);
Key projects in Scientific Research of Henan Provincial Education Department (13A180850)

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(Received:2014-02-28 Accepted:2014-03-23)

human glial cell derived neurotrophic factor (GDNF) gene fragment includes two exons (exon1+exon2). Two primer pairs were desigedned to amplify GDNF-1 (exon1) Forward-primer is 5'-GAAGATCTATGAAGTTATGGGATGTCGTG-3' *Bgl*II restriction enzyme cut site was added in this primer which is the outermost primers in the upstream and the primer reverse is 5'-TCTGGCATATTTGAGTCACTGCTCAG-3', Other two primer pairs were designed to amplify the exon2 (GDNF-2). Forward-primer is 5'-GAGCAGTGACTCAAATATGCCAGA-3' and the primer reverse is 5'-TTCCCGGGTCAGATACATCCACACCTTT-3'; *Sma*II restriction enzyme cut site was added in this 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 Neurotrophin3 (NT-3). Forward-long primer (5'-AACCATGTCCATCTTGTTTTATGTGATATTTC-3') was 4 bases longer than forward-short (5'-ATGTCCATCTTGTTTTATGTGATATTTCTCGC-3') at the 5' end. So did primer reverse-long (5'-GGCCGCTCATGTTCTTCCGATTT-3') than reverse-short (5'-GCTCATGTTCTTCCGATTTTTCTCGAC-3'). The underlined bases in 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 774 bp.

1.3 Construction of pIRES2-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 glial cell derived neurotrophic factor gene CDS (coding sequence) from genomic DNA. The procedure included four primers and three-step PCRs. human glial cell derived neurotrophic factor gene consists of two exons and the CDS contains 636 bps.

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 desigedned 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 the genomic DNA 0.5 μL (10ng). 2 × Prime STAR Max DNA Polymerase 10μL, RNase-Free Water 7.5 μL, upstream primer and downstream primer 2 μL. The reaction conditions were: 95 ℃ for 5min, 98 ℃ for 10 seconds, 55 ℃ for 5 seconds, and 72 ℃ for 5 seconds, 30cycles, finally, 72 ℃ maintained for 5 minutes. PCR product was purified by the PCR Purification Kit, then the PCR product and plasmid pIRES2-EGFP was cut by *Bgl*II and *Sma*II. After digested, 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, and including pIRES2-EGFP 2 μL, cDNA (GDNF) 8 μL, T4 DNA ligase 0.2 μL was added at 22 ℃ for 30 minutes. The DNA fragment was translated into *E.coli* DH5α and placed on LB plate (kanr) at 37 ℃ in incubator for 16 h. Monoclonal colony was picked up and shaken for 12-16 hours at 37 ℃ with a speed of 225r/minutes. The plasmid was extracted and identified by using *Bgl*II and *Bam*HI double enzyme digestion. The recombinant plasmid pIRES2-GDNF-EGFP was obtained.

1.4 Construction of pIRES2/ GDNF-NT-3

A simple and efficient method was developed for directional cloning of PCR products without any restriction enzyme digestion of the amplified sequence. Two pairs of primers were designed in which parts of two restriction enzyme recognition sequences were integrated, and the primers were used for two 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 end sensitivity of restriction enzymes.

1.4.1 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 mononuclear cells as templates. Both amplifications were subjected to 30 cycles of 94 ℃ for 30 s, 54 ℃ for 30 s and 72 ℃ for 1min, followed by a 5 min extension at 72 ℃ using 2× 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 2000UV Vis Spectrophotometers.

1.4.2 Cloning of PCR products pIRES2-GDNF-EGFP was digested with *Bst*XI and *Not*I, followed by phenol: chloroform extraction and ethanol precipitation. Meanwhile, each pooled NT-3 PCR products amplified with 2× Prime STAR Max DNA Polymerase were mixed, denatured at 94 ℃ for 4 min and re-annealed at 65 ℃ for 2 min. For a ligation reaction, the annealed NT-3 and linearized pIRES2-GDNF-EGFP (molar ratio equal to 4:1) were incubated with T4 DNA ligase at 16 ℃ for 3 h. For TA cloning, the two PCR products amplified by using EXTaq polymerase were mixed equimolarly and used to ligate with pMD18-T according to the manufacturer's instructions. The two ligation products were used to transform *Escherichia coli* DH5a $CaCl_2$ competent cells following standard method. Screening of the transformants was per formed by *Bst*XI plus *Not*I digestion following alkalinelysis plasmid preparation, and the recombinants were confirmed by DNA

sequencing(Fig. 1).

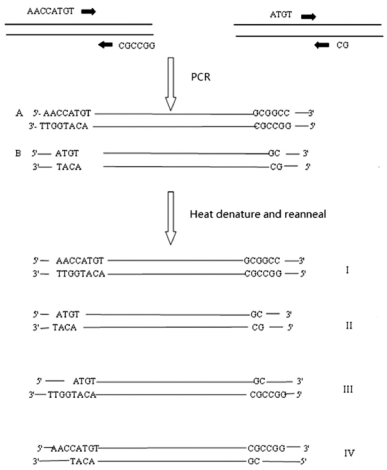

Fig. 1 Outline of twin PCR

Two parallel PCRs are performed, one with the forward-long and reverse-short primers. The other with the forward-short and reverse-long primers. The two PCR products, molecule I and II, are only different at the very ends as indicated. Equimolar of the two PCR products are mixed, heat-denatured and re-annealed. Four types of double-stranded DNA molecules with equal proportions are generated via random complementary pairing. 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 pIRES2-GDNF-EGFP digested with *Bst*XI and *Not*I.

1.5 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 ℃ 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 5×10^5cells/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 pIRES2-EGFP, pIRES2-GDNF/EGFP, and pIRES2-GDNF/NT-3 three vectors using Lipofectamine™ 2000 respectively for 2 h 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, Then they were observed under the inverted fluorescent microscope. Analysis of expression of the GDNF and NT-3 gene by RT-PCR. To detect the GDNF and NT-3 mRNA expression levels in HEK293 cells which either mock infected or infected with the pIRES2-EGFP, pIRES2-GDNF/EGFP, and pIRES2-GDNF/NT-3 three vectors respectively 3 days later, the GDNF and NT-3 expression was primarily assessed by means of RT-PCR. The methods of extracting total RNA and reverse transcription were same as the above. PCR amplification of these sequences from harvested cDNAs used these primers: GDNF, primer1 (forward), 5'-GAAGATCTATGAAGTTATGGGATGTCGTG-3' and pri- mer2 (reverse), 5'-TTCCCGGGTCAGATACATCCACACCTTT-3'. NT-3 primer3 (forward), 5'-AACCATGTCCATCTTGTTTTATGTGATATTTC-3'and primer4 (reverse), 5'-GGCCGCTCATGTTCTTCCGATTT-3'. β-Actin primer 5 (forward), 5'-AGCGGG AAATCGTGCGTGAC-3' and primer6 (reverse), 5'-CAAGAAA GGGTGTAACGCAACTA-3'.

1.6 Statistical analysis

Results expressed as mean± SD were recorded from the five experiments. The significance of differences was determined by one-way analysis of variance(ANOVA).

2 Results

2.1 Amplification of GDNF and NT-3 genes

GDNF and NT-3 genes were obtained from the genomic DNA of human peripheral blood mononuclear cells by PCR. The size of GDNF gene was 636 bp, the size of NT-3 gene was 774 bp (Fig. 2).

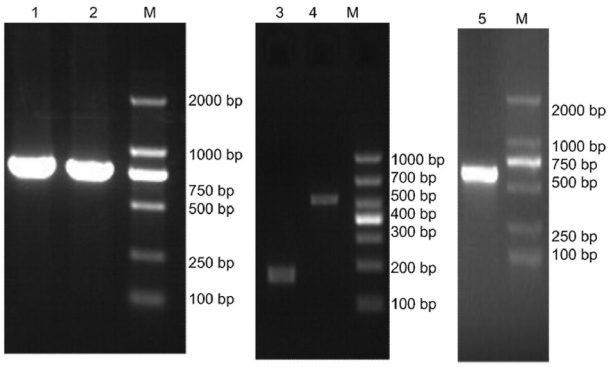

Fig. 2 The amplification of NT-3 and GDNF

M is Marker, Number 1 is NT-3(forward-short/reverse-long primer pairs)gene, Number 2 is NT-3 (forward-long/reverse-short primer pairs)gene. Number 3 is GDNF-1(exon 1) gene. Number 3 is GDNF-2 (exon 2) gene. Number 5 is GDNF (exon1+exon 2) gene.

2.2 Identification of plasmid pIRES2-GDNF-EGFP

The plasmid pIRES2-GDNF-EGFP was cut by *Bgl*II and *Bam*HI double enzyme. A gene fragment with 744 bp was obtained, which was in full agreed with GDNF gene(Fig. 3A).

2.3 Identification of plasmid pIRES2-GDNF-NT-3

The plasmid pIRES2-GDNF-NT-3 was cut by *Bgl*II and *Bam*HI double enzyme. And a fragment about 744 bp would be obtained. This indicated GDNF gene inserted into the plasmid pIRES2-GDNF-NT-3. The sequence of the plasmid pIRES2-GDNF-NT-3 was in accordance with gene sequence in Gene Bank (Fig. 3B).

2.4 RT-PCR analysis the expression of GDNF and NT-3

To illustrate mRNA expression by pIRES2-EGFP, pIRES2-GDNF/EGFP, and pIRES2-GDNF/NT-3 transduced HEK293 cells; we evaluated the expression of GDNF and NT-3 by RT-PCR analysis. RT-PCR was performed using GDNF-specific primers and the β-actin sequence as an internal

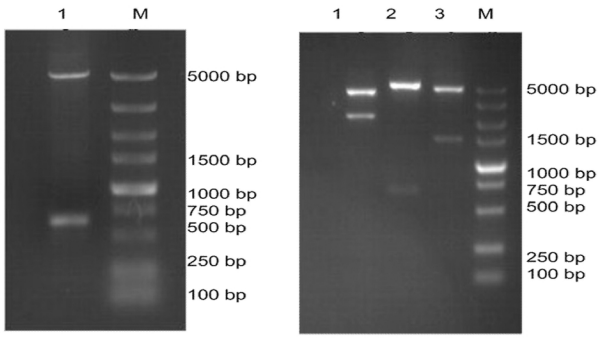

Fig. 3 A The identification of plasmid pIRES2-GDNF-EGFP. M is Marker, Number 1 is pIRES2-GDNF-EGFP cut by *Bgl*II and *Bam*HI double Enzyme

Fig. 3B The identification of plasmid pIRES2-GDNF-NT-3. M is Marker, Number 1 is pIRES2-GDNF-NT-3 cut by *Bgl*II and *Not*I, Number 2 is pIRES2-GDNF-NT-3 cut by *Bgl*II and *Bam*HI, Number 3 is pIRES2-GDNF -NT-3 cut by *Bam*HI and *Not*I

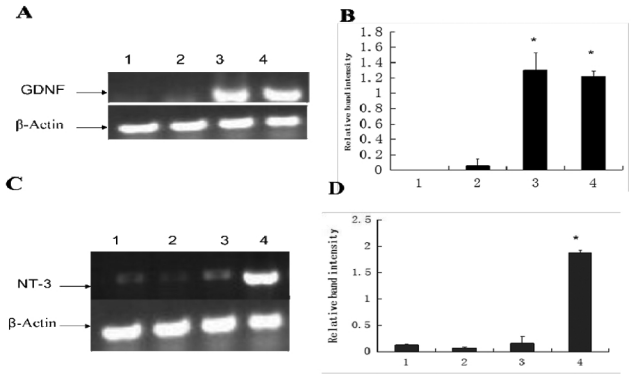

Fig. 4 A,B RT-PCR analysis of GDNF expression. GDNF (636bp), β-actin (551bp). A Lane1, NO transduced HEK293 cells; lane2, GFP Transduced HEK293 cells; lane 3, GDNF transduced HEK293 cells; lane 4, GDNF/NT-3 transduced HEK293 cells. B The mRNA expression level. The correspondence OD ratio of GDNF/β-actin. Data are mean ± 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 (774bp), β-actin (551bp)

C Lane 1, NO transduced HEK293 cells; lane 2, GFP transduced HEK293 cells; Lane 3, GDNF transduced HEK293 cells; lane 4, GDNF/NT-3 transduced HEK293 cells. D The mRNA expression level. The correspondence OD ratio of NT-3/β-actin. Data are mean ± SD(n=3). Compared with NO transduced HEK293 cells or transduced by pIRES2-EGFP.*P<0.01

standard. GFP expression was monitored in pIRES2-EGFP and pIRES2-GDNF/ EGFP transduced HEK293 cells by inverted fluorescence microscopy. Expression of GDNF mRNA was higher in either pIRES2-GDNF/EGFP or pIRES2-GDNF/NT-3 transduced HEK293 cells than that pIRES2-EGFP transduced HEK293 cells or negative control (Fig4. A, B). As shown above. Expression of NT-3 mRNA was higher in pIRES2-GDNF/NT-3- transduced HEK293 cells than the other three (Fig. 4 C, D). These results demonstrated that the GDNF and NT-3 had been introduced successfully into HEK293 cells by pIRES2-GDNF/ EGFP and pIRES2-GDNF/NT-3. After the HEK293 cells were transduced by pIRES2-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.

Table 1 Summary of cloning efficiencies using twin PCR and TA cloning

Vector [a]	Insert	Colonies[b]	percent with insertc
Linear pIRES2-GDNF-EGFP	PrimeSTARMax-amplified NT-3	149	85
pMD18-T	ExTaq-amplified NT-3	76	99
Circular pIRES2-GDNF-EGFP (1 ng)	——	428	——

Note: a Equimolar pMD18-T and *BstX*/*Not* I cleaved pIRES2-GDNF-EGFP; b Only white colonies were recorded in TA cloning; c 20 colonies from each transformation were randomly selected for analysis.

3 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 which were complimentary to restriction enzyme digested vector [11]. As shown in Fig.1, PCR products reassembled via complimentary pairing after heat-denaturing and re-annealing. Four types of molecules with equal proportions were created, two of which were blunt-ended and another two were sticky-ended. Theoretically, half of the sticky-ended products are suitable for a certain ligation. In practice, when the re-annealed mixture was used to ligate with prelinearized pIRES2-GDNF-EGFP and transform DH5a, 149 colonies were obtained with 85% of the screened colonies containing the correct NT-3 insert (Table.1). Admittedly, only 25% re-annealed products could be inserted into linear vector, which means more PCR products will be needed for the twin PCR approach than TA cloning. But the absolute quantity of the insert required for a twin PCR is easy to get by standard PCR, and the cloning efficiency for those joinable DNA fragments is higher than that of TA cloning. If PEG was used in the ligation reaction like TA cloning, the cloning efficiency is likely to be improved. Using twin PCR strategy, hybridized PCR products flanked with all kinds of sticky ends that are compatible with almost all restriction enzymes can be generated based solely on primer designing and PCR amplification. For example, if the introduced deoxynucleotides dC and dGGCCGC at the 5' end of two reverse primers were replaced by dG and dAACCATG separately, and the two parallel PCRs were performed using either forward-long/reverse-long or forward-short/reverse-short primer sets. After heat-denaturing and re-annealing treatment, 25%

products would be 5' over hanged at the upstream and 3'over hanged at the downstream, which could be inserted into pIRES2-GDNF-EGFP at the BstXI and Not I sites. Since no restriction enzyme digestion is required for the PCR products, twin PCR overcomes the problems 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 have more opportunities to contain kinds of restriction enzymes sites.

In this article, NT-3 was directly inserted into pIRES2-GDNF- EGFP at the *Bst*XI and *Not* I sites, despite the exiting *Bst*XI site within its coding sequence (Data not shown). Moreover, those more efficient and economical enzymes, such as *Bst*XI and NotI, could always be chosen for vector digestion. It is not any longer necessary to buy so many Kinds of restriction enzymes for cloning. Meanwhile, our cloning method has other advantages. It is so simple that the whole procedure, including PCR, enzyme digestion, ligation and transformation could be completed in 8h, leading to the substantial saving in time, cost and labor. In addition, interested inserts are amplified using proof-reading DNA polymerases, which provide a faithful guarantee for the PCR products. These advantages make it a universal technique for the directional cloning of PCR products.

Mesenchymal stem cells (MSCs) are a population of self-renewing, multipotent cells that are able to differentiate along several committed phenotypes including osteogenic, chrondogenic, adipogenic lineages in response to stimulation by multiple environmental factor. Many cytokines and transcription factors play an important role in this procedure [12].

The present study investigated neuroprotective effects of glial cell line-derived neurotrophic factor (GDNF), a distant member of the transforming growth factor-beta (TGF-beta) superfamily, following moderate contusive spinal cord injury (SCI) in adult rats[13]. Glial cell line-derived neurotrophic factor (GDNF), which has been demonstrated to be the most potent neurotrophic factor for the proliferation, differentiation, and survival of spinal motoneurons, exhibits very good therapeutic potential for ALS[14]. Systemic administration of GDNF as are combinant protein to ALS patients, however, is not beneficial, because of its short plasma half-life and poor access to motoneurons on the one hand, and, on the other hand, because of its severe side effects that prevent its administration at an adequate dose [15]. If ways that make continuous and motoneuron-confined delivery of GDNF possible are established, the disadvantages of its systemic administration will be overcome. Gene therapy involving the injection of a vector encoding a gene for GDNF into skeletal muscles will be a good candidate for such a method [16] Following dorsal root injury, GDNF proved to be the most effective neurotrophic factor in stimulating axonal growth across the dorsal root entry zone into the spinal cord white matter [17]. GDNF-transduced mesenchymal stem cells, grafted into a spinal cord dorsal hemi-lesion site, also greatly enhanced sensory axon growth [18].

To date, NT-3 is the only neurotrophic factor that has been identified to promote the growth of corticospinal axons after spinal cord injury [19]. When delivered as a single injection into the lesioned spinal cord, NT-3 promoted sprouting of corticospinal axons [20]. When delivered to the spinal cord using a continuous ex vivo gene delivery approach, NT-3 promoted the growth of corticospinal axons and achieved partial functional recovery [21]. In another study, a 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 [22]. Also, infusion of NT-3 into the intrathecal space promoted regeneration of dorsal column sensory axons into and beyond a site of spinal cord injury [23]. Thus, a variety of axonal populations of the spinal cord respond to neurotrophic factors. The elucidation of 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 GDNF and NT-3 genes were inserted into a bicistronic eukaryotic expression vector. The plasmid pIRES2-GDNF-NT-3 was constructed successfully. The plasmid would be transfected into Mesenchymal stem cells in the next experiment and the GDNF and NT-3 genes would express in the cells, it is possible that combined gene therapy of both genes might have more significant effects on neuron regeneration than single gene alone.

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pIRES2-GDNF-NT-3 真核表达载体的构建与鉴定 *

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摘要 目的：采用一种简便和高效的方法构建双基因共表达载体 pIRES2-GDNF-NT-3。**方法：**人胶质细胞源性神经营养因子和神经营养素 3 是采用 PCR 的方法从人外周血单个核细胞的基因组 DNA 中获取，将人胶质细胞源性神经营养因子的 cDNA 片段插入到 pIRES2-EGFP 多克隆位点构建成为 pIRES2-GDNF-EGFP. 神经营养素 3 cDNA 片段通过替换 EGFP 的方式插入到 pIRES2-GDNF-EGFP 中构建成为 pIRES2-GDNF-NT-3 双基因共表达载体。**结果：**人胶质细胞源性神经营养因子和神经营养素 3 被克隆，通过测序和酶切鉴定的得知与基因库报道序列一致。**结论：**人神经生长因子和神经营养素 3 双基因真核表达载体成功构建，它提供了一个新的表达系统，为进一步研究双基因的功能奠定了基础。

关键词：胶质细胞源性神经营养因子；神经营养素 3 ；真核双表达载体；内部核糖体进入位点

中图分类号：Q784；Q75 **文献标识码：**A **文章编号：**1673-6273(2014)31-6056-03

* 基金项目：河南省重大科技攻关项目(122101310100)；新乡医学院重点领域招标课题(ZD2011-16)；
河南省教育厅科学技术研究重点项目(13A180850)

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（收稿日期：2014-02-28 接受日期：2014-03-23）