Science in China Series C: Life Sciences

www.scichina.com
life.scichina.com
www.springerlink.com

The targeting expression of the vascular endothelial growth factor gene in endothelial cells regulated by HRE.ppET-1

ZHENG XiangRong†, ZHANG ShangShang, YANG YuJia, WANG Xia, ZHONG Le & YU XiaoHe

Department of Pediatrics, Xiangya Hospital, Central South University, Changsha 410008, China

The success of gene therapy depends largely on the efficacy of gene delivery vector systems that can deliver genes to target organs or cells selectively and efficiently with minimal toxicity. Here, we show that by using the HRE.ppET-1 regulatory element, we were able to restrict expression of the transgene of vascular endothelial growth factor (VEGF) to endothelial cells exclusively in hypoxic conditions. Eukaryotic expression vectors such as pEGFP-HRE.ppET-1, pcDNA3.1-VEGF+Pa, pcDNA3.1-ppET-1+EGF+Pa, and pcDNA3.1-HRE.ppET-1+VEGF+Pa were constructed by using a series of nuclear molecule handling methods like PCR, enzyme digestion. The recombinant vectors were transfected into HUVEC cells and HL7702 cells by the lipofectin method. GFP expression was observed with a fluorescence microscope to validate the specificity of expression in endothelial cells under the regulation of HRE.ppET-1 element. Cobalt chloride (final concentration 100 μmol/L) was added to the medium to mimic hypoxia *in vitro*. After transfection of vectors, the expression of VEGF mRNA was detected by RT-PCR, and the expression of VEGF was detected by Western blotting and ELISA methods under normoxia and hypoxia, respectively. The cell proliferation rate was detected by the MTT test. The expression of GFP revealed that the exterior gene was transcripted effectively in endothelial cells regulated by the HRE.ppET-1 element, while the expression of GFP was very weak in nonendothelial cells. The results of RT-PCR, Western blotting and ELISA showed that VEGF gene expression in the pcDNA3.1-HRE.ppET-1+VEGF+Pa group and in the pcDNA3.1-ppET-1+VEGF+Pa group was higher in hypoxia than it was in normoxia (P<0.05). The MTT test showed that the proliferation rate of HUVEC transfected with HPVA under hypoxia exceeded that of the control group. We conclude that the HRE.ppET-1 element was expressed specifically in endothelial cells, and can increase the expression of VEGF in hypoxia and stimulate proliferation of endothelial cells. Taking advantage of these facts could greatly improve the efficiency of gene therapy. The vector would be valuable for various gene transfer studies targeting endothelial cells.

hypoxia-responsive element, human preproendothelin-1 promoter, vascular endothelial growth factor, endothelial cells

Vascular endothelial growth factor (VEGF) is best known for its role in angiogenesis. Recent insights into the role of VEGF in a variety of neurological disorders suggest that it is a promising therapeutic target in ischemic brain damage[1–3], because it can promote ischemic capillary formation, increase neurogenesis of the subependymal region and improve neurological function[4]. However, due to the lack of selectivity toward target cells, its application in clinical practice is limited. Ensuring that the VEGF gene is strictly limited to the target cells is the key to improving the therapeutic effect. Transcription of the VEGF gene is activated un-

Received May 16, 2008; accepted August 5, 2008
doi: 10.1007/s11427-008-0116-7
†Corresponding author (email: zxr_168@126.com)
Supported by the National Natural Science Foundation of China (Grant No. 30500541)

der hypoxia. The interaction of a transcriptional complex termed hypoxia-inducible factor-α (HIF-1α) with its cognate DNA recognition site, typically 5′-TACG TGC-3′, is known as the hypoxia response element (HRE)[5], which increases transcription of the VEGF gene. We examined the possibility that HRE could be used effectively as a trigger to regulate VEGF expression under hypoxia.

It has been shown that the human preproendothelin-1 (ppET-1) gene is expressed specifically in vascular endothelial cells and neurons in brain tissue[6]. Analysis of deficiency mapping found that ppET-1 gene expression was promoted through HIF-1α binding HRE specifically under hypoxia. We used the ppET-1 gene promoter to develop highly efficient, endothelial cell-specific expression vectors.

In this study, we constructed a novel eukaryotic expression vector whose promoter is the HRE.ppET-1 regulatory element. The vector drives a high level of VEGF gene expression in endothelial cells, and the specificity is further enhanced in conditions *in vitro* that mimic natural hypoxia. The vector is a potentially useful tool for selective gene therapy in ischemic brain damage.

1 Materials and methods

1.1 Materials

Plasmid T-VEGF and *Escherichia coli* JM109 were kindly provided by the National Laboratory of Medical Genetics of China in Central South University. pEGFP-N1 and pcDNA3.1 were purchased from Promega. Human umbilical vein endothelial cell line HUVEC and liver cell line HL7702 were purchased from the China Center for Type Culture Collection (CCTCC).

Various enzymes (restriction endonuclease, polymerase, and ligase) were purchased from the Takara. Lipofectamine 2000 was purchased from Invitrogen, reverse transcription kits were purchased from Promega, VEGF antibody was purchased from Santa Cruz, and the VEGF ELISA kit was obtained from Jingmei Biotech.

1.2 Plasmid construction

The adhesive ends of *Nhe*I and *Xba* were connected to their corresponding pcDNA3.1 vector after direct annealing with HRE oligonucleotides. Using human gDNA, T-VEGF plasmid, and pcDNA3.1 as template, the ppET-1 promoter, VEGF and poly(A) were amplified by PCR. The products were purified by extraction with phenol/chloroform, digested directly by the corresponding endonuclease, and then connected to the pcDNA3.1 vector. The eukaryotic expression vectors pcDNA3.1-VEGF+Pa(VA), pcDNA3.1-pET+VEGF-1+Pa(PVA), and pcDNA3.1-HRE ppET-1+ VEGF+Pa(HPVA) were constructed through re-connection and digestion. Finally, HRE.ppET-1 was cut off and connected to the pEGFP-N1 vector to construct pEGFP-HP. These vectors were identified by enzymatic digestion and sequencing. The primers used are shown in Table 1 and a conceptual diagram of the vector is shown in Figure 1.

1.3 Cell culture, transfection and grouping

HUVEC cells were grown in M199 medium with 10% FBS, and HL7702 cells were grown in 1640 medium with 10% FBS; both were incubated at 37℃, in a 5% CO_2 atmosphere. Two kinds of cells were seeded into 6-well cell microplates at a density of 2×10^5 cells/well. When the cell confluence was approximately 80%, transfection of pEGFP-HP and the pEGFP-N1 empty plasmid was done with the Lipofectamine 2000 system

Table 1 The primers used for amplification

Primer	Sequence (from 5′ to 3′)	Site of digestion
HRE-up	CTAGCCCACAGTGCATACGTGGGCTCCAACAGGTCCTCTTT	*Nhe* I
HRE-lp	CTAGAAAGAGGACCTGTTGGAGCCCACGTATGCACTGTGGG	*Xba* I
ppET-up	ATTGCGGCCGCTCTGAAGTTAGCAGTGATTTCCTTTCG	*Not* I
ppET-lp	CCGGAATTCTCTGAAAAAAAGGGATCAAAAACCTC	*Eco*R I
ppET-lp	CCGGAATTCTCTGAAAAAAAGGGATCAAAAACCTC	*Eco*R I
ppET-lp	CCGGAATTCTCTGAAAAAAAGGGATCAAAAACCTC	*Eco*R I
polyA-up	CGCGGATCCTGTGCCTTCTAGTTGCCAGCC	*Bam*H I
polyA-lp	CGGGGTACCACCGCATCCCCAGCATGCCT	*Kpn* I
VEGF-up	CCGGAATTCATGAACTTTCTGCTGTCTTGGG	*Eco*R I
VEGF-lp	CGCGGATCCTCACCGCCTCGGCTTGTCA	*Bam*H I

according to the manufacturer's instructions. At 24 h after transfection, expression of green fluorescent protein (GFP) was detected by fluorescence microscopy. Transfection of the plasmids VA, PVA and HPVA to HUVEC cells was done in the same way. The blank control group was not transfected with a plasmid. After transfection, HUVEC cells and blank control group were processed with normoxia and hypoxia, respectively; therefore, they can be divided into eight groups: VA+ normoxia, VA+hypoxia, PVA+normoxia, PVA+hypoxia, HPVA+normoxia, HPVA+hypoxia, blank+normoxia, and blank+hypoxia. In the hypoxia groups, cobalt chloride was added to a final concentration of 100 μmol/L as a simulation of natural hypoxia[7].

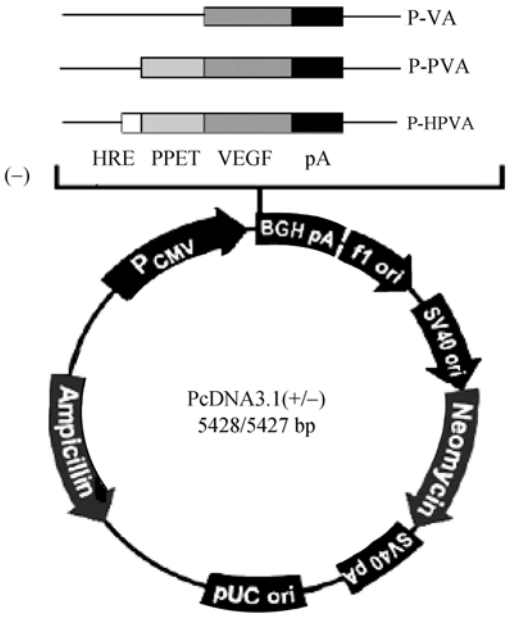

Figure 1 A conceptual diagram of the vector. The promoter of VA was CMV in the vector origin; the CMV promoter was removed by an incision enzyme in the vector of PVA and HPVA.

1.4 RT-PCR analysis for VEGFmRNA

At 48 h after transfection, the total cellular RNA of each group was isolated with Trizol® reagent. Using total RNA as a template, reverse transcription was done according to the Reverse Transcription System:VEGF (5′-C CGGAATTCATGAACTTTCTGCTGTCTTGGG-3′; 5′-CGCGGATCCTCACCGCCTCGGCTTGTCA-3′) was amplified using the reverse transcription product as a template. β-Actin was amplified as a control. The amplification conditions were 28 cycles of 95℃ for 5 min, 94℃ for 30 s, 56℃ for 30 s, 72℃ for 30 s, and then 72℃ for 10 min. The PCR product was examined by electrophoresis in a 1.5% agarose gel.

1.5 Western blot analysis

Western blot analysis was used to detect semi-quantitative levels of the VEGF protein. After 24 h of conventional culture following transfection, cells of each group were cultured in serum-free medium for 24 h. The supernatants of the cell cultures were collected, then separated by SDS-PAGE (10% polyacrylamide gel). Samples were transferred onto nitrocellulose membranes and incubated overnight at 4℃ in blocking solution. The membranes were exposed to polyclonal rabbit anti-human anti-VEGF antibodies (1∶500) for 1 h at room temperature. After washing, the membranes were incubated with anti-rabbit antibody HRP (1∶5000) for 1 h at room temperature, followed by three washes of 15 min each in PBS, 0.1% Triton. The presence of antibody was detected with an enhanced chemiluminescence (ECL) kit. The tubulin proteins of clearage cells were used as an internal control.

1.6 ELISA for VEGF protein content in cell culture supernatant

The VEGF protein content was quantified in the supernatant of each group using an ELISA kit according to the manufacturer's instructions. A standard curve for substrate fluid versus standard preparation fluid was constructed. The values of A at 492 nm were determined, and the concentrations of VEGF were calculated via the standard curve.

1.7 The MTT test for cell proliferation rate

The cell proliferation rate was determined by the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) test. HUVEC and U251 cells were loaded in triplicate into 96-well culture plates at ~ 1000 cells/well. HPVA was transfected into HUVEC and U251 cells, which were processed under normaxia and hypoxia, respectively, and nontransfected cells under normoxia was used as the control group, and incubated for 7 days. Every day, 20 μL of 0.5% MTT was added to each well, the plate was incubated for 4 h, fluid was removed from each well by suction, 150 μL of DMSO was added to each well, the plate was shaken for 10 min, and then A at 490 nm was determined. Average values were calculated and used in the following equation:

$$\text{Cell proliferation rate} = (A_s - A_b) / (A_c - A_b) \times 100\%,$$

where A_s is the A value of sample group, A_b is the A value of the blank group, and A_c is the A value of the control group.

1.8 Statistical analysis

Statistical analysis was performed using SPSS 11.5 software. Measurement data are presented as mean ± SD. Analysis of variance (ANOVA) was used for examining differences between groups.

2 Results

2.1 GFP expression

The viability of cells was 80% at 24 h after transfection, and GFP expression of HUVEC cells and HL7702 cells transfected with pEGFP-HP was observed with a fluorescence microscope. The number of HP-mediated HUVEC positive cells was higher than that of HL7702 cells, but the fluorescence intensity was weaker compared with the empty plasmid, and exposure for a longer time was required (Figure 2). The transfection efficiency was 18.7%, <5%, 25.4% and 37.2%, as shown in Figure 2(a)—(d), respectively. This showed that HRE.ppET-1 can activate exogenous gene expression specifically in endothelial cells, but does not have a strong transcription effect.

2.2 RT-PCR assay

After transfection with plasmids, HUVEC cells were further divided into normoxia and hypoxia groups. After incubation for 48 h, total RNA was extracted, and RT-PCR was used to examine the VEGFmRNA. β-Actin of 120 bp acted as an endogenous control for semi-quantification of VEGF expression. Figure 3(a) and (b) shows the results of electrophoresis of the RT-PCR products; the amount of VEGF expressed in the HPVA+hypoxia group was the greatest, and was significantly higher than that in other groups (P<0.05).There was no significant difference between the VA+normoxia and VA+hypoxia groups, but the amounts of VEGF expressed in the PVA+hypoxia, VA+hypoxia and VA+normoxia groups were significantly higher than that in the PVA+normoxia group (P<0.05). This suggested that the transcription efficiency of the ppET promoter under normoxia was weak. The levels of VEGF mRNA in the hypoxia control group also increased, which indicated that the cells responded to the hypoxia directly.

2.3 Western blot assay

Figure 4 shows the Western blot results with tubulin as the internal control. The results showed that the 40 kDa VEGF protein was detected in all groups except blank+hypoxia, blank+normoxia, and the levels of gene expression at translation and transcription were basically equal. This may because the concentrations of collected protein were low. The VEGF protein was not detected in the blank+hypoxia, blank+normoxia groups, in which VEGF mRNA was detected by RT-PCR.

2.4 ELISA

The ELISA results showed that the concentrations of VEGF in the VA+hypoxia and HPVA+hypoxia groups were the highest, and were considerably higher compared with those in the other groups (Figure 5; P<0.01). There was no significant difference between the VA+hypoxia and HPVA+hypoxia groups, which indicated that the HRE.ppET-1 regulatory element could enhance the downstream gene expression efficiently under hypoxia. At the same time, the concentrations of VEGF under hypoxia were significantly higher than those under normoxia (P<0.05), suggesting that endothelial cells produced their own response to the increased expression of HRE and VEGF.

2.5 MTT test

In the first day after transfection with HPVA, the proliferation of cells in each group was slow, perhaps because

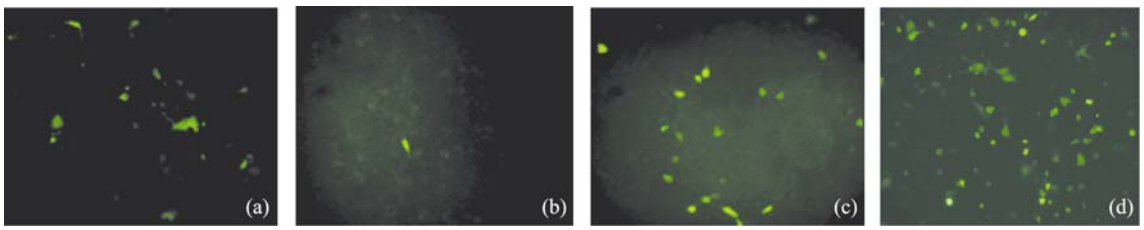

Figure 2 (a) Expression of GFP in HUVEC cells at 24 h after transfection with pEGFP-HP. (b) Expression of GFP in HL7702 cells at 24 h after transfection with pEGFP-HP. (c) Expression of GFP in HUVEC cells at 24 h after transfection with pEGFP-N1. (d) Expression of GFP in HL7702 cells at 24 h after transfection with pEGFP-N1.

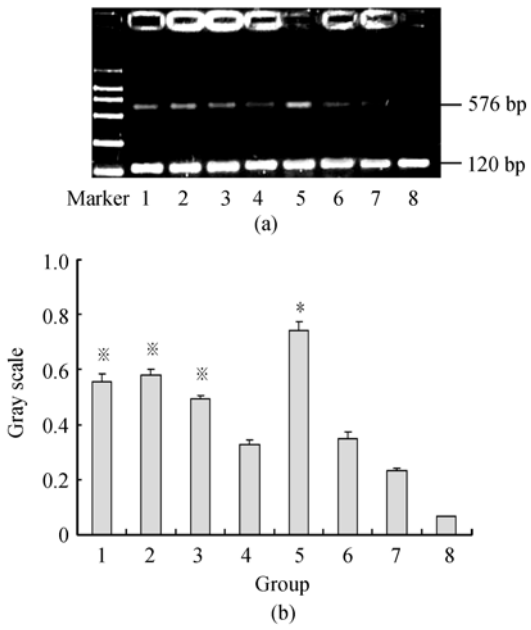

Figure 3 (a) Expression of VEGFmRNA by RT-PCR. (b) Semiquantitative results of VEGF mRNA expression. 1, VA+hypoxia; 2, VA+normoxia; 3, PVA+hypoxia; 4, PVA+normoxia; 5, HPVA+hypoxia; 6, HPVA+ normoxia; 7, blank+hypoxia; 8, blank+normoxia. The expression of VEGFmRNA in the HPVA+hypoxia group was higher than that in the other groups (*, P<0.05), while the expression of VEGFmRNA in the VA+hypoxia, VA+normoxia, and PVA+hypoxia groups was higher than that in the PVA+normoxia group (※, P<0.05).

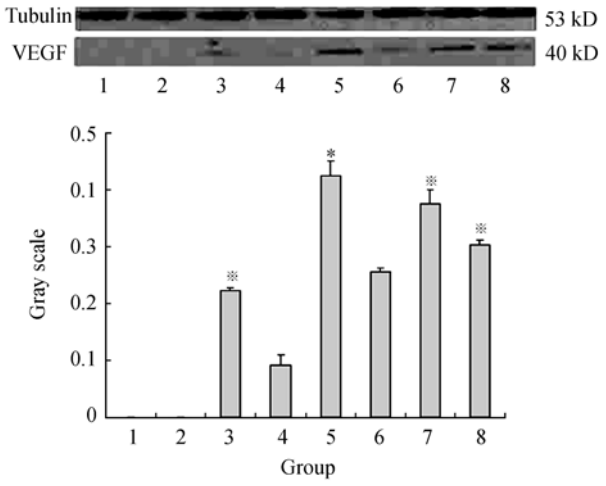

Figure 4 Expression of VEGF165 protein as determined by Western blotting. 1, Blank+hypoxia; 2, blank +normoxia; 3, PVA+hypoxia; 4, PVA+normoxia; 5, HPVA+hypoxia; 6, HPVA+normoxia; 7, VA+hypoxia; 8, VA+normoxia. In each group, the difference of protein level mostly matches the difference of transcriptional level. *, P<0.05,HPVA+hypoxia vs HPVA+normoxia; ※, P<0.05, VA+hypoxia, VA+normoxia and PVA+hypoxia vs PVA+ normoxia.

of a certain amount of toxicity in the transfection process. After changing the liquid, cells began to proliferate on the second day, and reached the level in the corresponding control group. The proliferation of HUVEC transfected with HPVA under hypoxia exceeded that of the corresponding control group, suggesting that VEGF expression under hypoxia stimulates HUVEC proliferation. However, after a few days under hypoxia, the cells did not maintain the rate of proliferation, which began to decline, perhaps because expression of VEGF cannot be sustained, and prolonged hypoxia caused damage to cells. There was no significant difference in proliferation rate between the two kinds of cells in the last 4 days. The cell growth curve is shown in Figure 6.

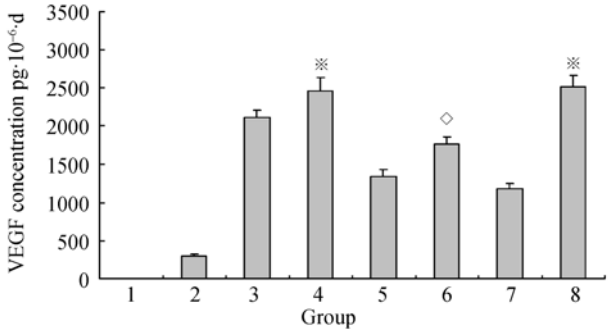

Figure 5 The amount of VEGF protein in the cellular supernatant of each group. 1, Blank+normoxia; 2, blank+hypoxia; 3, VA+normoxia; 4, VA+hypoxia; 5, PVA+normoxia; 6, PVA+hypoxia; 7, HPVA+normoxia; 8, HPVA+hypoxia. ※, P<0.01, compared with each other group; ◇, P<0.05, compared with group PVA+normoxia.

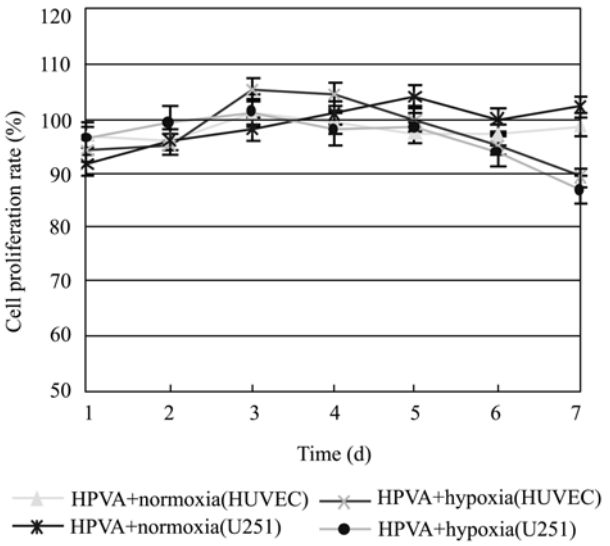

Figure 6 Cell growth curve. Time is on the horizontal (x) axis, cell proliferation rate (%) is on the vertical (y) axis.

3 Discussion

Transcriptional targeting in gene therapy has become a hot topic. Using cellular tissue-specific promoters or

gene regulation components that can be expressed excessively in some pathological states is expected to guide the high-level expression of a theoretical gene to a particular tissue[8,9]. The objective of gene therapy is to achieve efficient and punctual expression of a theoretical gene in a particular cell type[10,11]. The objective of the present study was to increase transcriptional targeting in gene therapy. We demonstrated that the ppET-1 promoter plus HRE drive the expression of the VEGF gene to be specifically in endothelial cells, confirming that the HRE.ppET-1 regulatory element increased VEGF gene expression markedly under hypoxia.

Under hypoxia, transcription of the VEGF gene is activated to maintain systemic and local oxygen homeostasis. An important mediator of these responses is the interaction of a transcriptional complex termed the hypoxia-inducible factor-1α (HIF-1α) with its cognate DNA recognition site, typically 5′-TACGTGC-3′, which is known as the hypoxia response element (HRE). HRE is a conserved DNA sequence, which exists in hypoxia regulatory genes. HRE is sensitive to hypoxia, and it is essential for the expression of VEGF in hypoxia[12].

It has been shown that the expression of a heterologous promoter that had been modified by HRE is augmented in hypoxia[13]. By joining HRE with promoters such as AFP and Sv40 to drive a reporter gene, the capacity for enhanced gene expression was acquired under hypoxia. Binley et al.[14] constructed a replication-defective recombinant adenovirus in which transcription of the *lac*Z gene was controlled by HRE, and confirmed that the expression of *lac*Z in infected cells was at only a very low level at physiological oxygen concentrations, while it increased rapidly under hypoxia. At present, most studies concerning HRE promoters in gene therapy are confined to gene therapy of a tumor[15,16], as most solid tumors commonly have severe hypoxia, and hypoxia is an important pathophysiological factor in the pathogenesis of ischemic brain damage[17]. Therefore, we used an HRE-modified promoter in our system. It is expected to overcome the traditional shortcomings of low efficiency in gene therapy, and thereby enhance the effectiveness of gene therapy.

In the process of seeking to increase expression of the VEGF gene, it was hoped that the VEGF gene could be targeted to vascular endothelial cells under hypoxia, and expressed efficiently and controllably. Study found that ppET-1 was expressed specifically in the vascular endothelial cells in brain[18], and as a neuromodulator or neurotransmitter, it participated in the development of neurons. It was produced when cells were stimulated by injury, hypoxia, ischemia, inflammation, etc. By using the ppET-1 gene promoter, Cho et al.[7] constructed an effective endothelial cell-specific expression vector. Compared with the control group for the CMV promoter, expression of the reporter gene in endothelial cells was enhanced 7.9-fold, and further enhanced 1.6-fold under hypoxia. This indicated that ppET-1 could cause the object gene to be expressed efficiently in endothelial cells *in vitro*. In this study, at 24 h after transfection, expression of GFP by HUVEC and HL7702 cells transfected with pEGFP-HP was observed with a fluorescence microscope. The number of HRE.ppET-1-mediated HUVEC positive cells was higher than that of HL7702 cells, and the transfection efficiency of HUVEC was increased significantly compared with HL7702. This suggested that HRE.ppET-1 can express an exogenous gene specifically in endothelial cells.

The results of this study have verified for the first time that a complex of HRE and the ppET-1 regulatory element can effectively mediate the expression of an exogenous gene in endothelial cells, while the transfection efficiency was very low in nonendothelial cells. Under hypoxia, the tranfection efficiency was increased markedly and could stimulate proliferation of endothelial cells. There are reports that copies of HRE are correlated closely to the activity of the promoter. Shen et al.[19] showed that the hypoxia sensitivity of nine copies of HRE tandem repetitive sequence (TRS) was greatest. This study used a single-copy HRE sequence that included the recognition sequence of HIF-1α and its own core sequence, which demonstrated an enhancement effect under hypoxia. In order to further enhance the strength of expression of the VEGF gene in the next step of this research project, we will use multiple copies of the HRE sequence. In addition, the low level of transfection efficiency in the nonviral vector plasmid largely restricted the feasibility of VEGF gene therapy, and we will attempt to develop a viral vector to carry out transfection both *in vitro* and *in vivo*.

Here, we demonstrated that our newly developed vector, whose promoter was the HRE.ppET-1 regulatory element, expressed the exogenous VEGF gene efficiently in endothelial cells *in vitro*. This vector can also increase the expression of VEGF under hypoxia, and

stimulate proliferation of endothelial cells, which could be used to greatly improve the efficiency of gene therapy. The vector would be valuable for various gene transfer studies targeting endothelial cells.

1 Lambrechts D, Carmeliet P. VEGF at the neurovascular interface: Therapeutic implications for motor neuron disease. Biochim Biophys Acta, 2006, 1762(11-12): 1109－1121

2 Meng H, Zhang Z, Zhang R, et al. Biphasic effects of exogenous VEGF on VEGF expression of adult neural progenitors. Neurosci Lett, 2006, 393(2-3): 97－101

3 Wang Y, Jin K, Mao X, et al. VEGF-overexpressing transgenic mice show enhanced post-ischemic neurogenesis and neuromigration. J Neurosci Res, 2007, 85(4): 740－747

4 Zhu W, Mao Y, Zhou L F. Reduction of neural and vascular damage by transplantation of VEGF-secreting neural stem cells after cerebral ischemia. Acta Neurochir Suppl, 2005, 95: 393－397

5 Ramanathan M, Pinhal-Enfield G, Hao I, et al. Synergistic up-regulation of vascular endothelial growth factor (VEGF) expression in macrophages by adenosine A2A receptor agonists and endotoxin involves transcriptional regulation via the hypoxia response element in the VEGF promoter. Mol Biol Cell, 2007, 18(1): 14－23

6 Naidoo V, Naidoo S, Mahabeer R, et al. Cellular distribution of the endothelin system in the human brain. J Chem Neuroanat, 2004, 27(2): 87－98

7 Cho J, Lim W, Jang S, et al. Development of an efficient endothelial cell specific vector using promoter and 5'untranslated sequences from the human preproendothelin-1 gene. Exp Mol Med, 2003, 35(4): 269－274

8 Boeckle S, Wagner E. Optimizing targeted gene delivery: chemical modification of viral vectors and synthesis of artificial virus vector systems. AAPS J, 2006, 8(4): E731－42

9 van Gaal E V, Hennink W E, Crommelin D J, et al. Plasmid engineering for controlled and sustained gene expression for nonviral gene therapy. Pharm Res, 2006, 23(6): 1053－1074

10 Lyon A R, Sato M, Hajjar R J, et al. Gene therapy: targeting the myocardium. Heart, 2008, 94(1): 89－99

11 Tanaka T, Kuroki M, Hamada H, et al. Cancer-targeting gene therapy using tropism-modified adenovirus. Anticancer Res, 2007, 27(6A): 3679－3684

12 Jeong C H, Lee Y M, Choi K S, et al. Hypoxia-responsive element-mediated soluble Tie2 vector exhibits an anti-angiogenic activity *in vitro* under hypoxic condition. Int J Oncol, 2005, 26(1): 211－216

13 Jiang B, Dong H, Zhang Z, et al. Hypoxic response elements control expression of human vascular endothelial growth factor(165) genes transferred to ischemia myocardium in vivo and in vitro. J Gene Med, 2007, 9(9): 788－796

14 Binley K, Askham Z, Martin L, et al. Hypoxia-mediated tumour targeting. Gene Ther, 2003, 10(7): 540－549

15 Liu J, Harada H, Ogura M, et al. Adenovirus-mediated hypoxia-targeting cytosine deaminase gene therapy enhances radiotherapy in tumour xenografts. Br J Cancer, 2007, 96(12): 1871－1878

16 Matzow T, Cowen R L, Williams K J, et al. Hypoxia-targeted over-expression of carboxylesterase as a means of increasing tumour sensitivity to irinotecan (CPT-11). J Gene Med, 2007, 9(4): 244－252

17 Li L, Qu Y, Li J, et al. Relationship between HIF-1alpha expression and neuronal apoptosis in neonatal rats with hypoxia-ischemia brain injury. Brain Res, 2007, 1180: 133－139

18 Varda-Bloom N, Shaish A, Gonen A, et al. Tissue-specific gene therapy directed to tumor angiogenesis. Gene Ther, 2001, 8(11): 819－827

19 Shen F, Su H, Fan Y, et al. Adeno-associated viral-vector-mediated hypoxia-inducible vascular endothelial growth factor gene expression attenuates ischemic brain injury after focal cerebral ischemia in mice. Stroke, 2006, 37(10): 2601－2606