The construction of siRNA plasmid targeting mouse HIF-1 α and *in vitro* study of its inhibition effect

Zhen-Yu DING², Ze-Gui LI¹, Yi-Zhan XING⁴, Hua JI^{4,*}, Hong-Li LI⁴, Zhi-Jie CHANG³

¹Department of pharmacognosy, College of pharmacy, Third Military Medical University, Chongqing 400038, China

²Department of Oncology, General Hospital of Shenyang Military Region, Shenyang 110016, China

³Institute of genome, College of Medicine, Tsinghua University, Beijing 100084, China

⁴Department of Histology and Embryology, College of Medicine, Third Military Medical University, Chongqing 400038, China

Abstract: Objective To construct effective RNA-interference plasmids targeting mouse HIF-1 α gene and testify their effects and specificities in interfering HIF-1 α expression. **Methods** Three RNA-interference plasmids targeting mouse HIF-1 α gene, pBS/U6/HIF-1 α -siRNAI~III, were constructed and identified using double digestion method in the present study. RT-PCR, immunostaining and western blotting were employed to detect the expression alterations of HIF-1 α in 293T cells following transfections of the three plasmids, respectively. The interference effect of pBS/U6/HIF1 α -II in SH-SY5Y cell line was further investigated. **Results** All the three RNA-interference plasmids, especially pBS/U6/HIF1 α -II, showed significant inhibition in HIF-1 α expression in 293T cell line. pBS/U6/HIF1 α -II could also inhibit HIF-1 α expression in SH-SY5Y cell line, in a dose-dependent way. **Conclusion** Plasmid pBS/U6/HIF1 α -II constructed in our study can effectively and specifically inhibit HIF-1 α expression, and its role in neural tube development and dysfunction will be further investigated. Construct of pBS/U6/HIF1 α -II plasmid will provide a useful tool to study the role of HIF-1 pathway in embryogenesis, oncogenesis and ischemia development.

Keywords: HIF-1 α ; RNA interference; pBS/U6/HIF-1 α -siRNA

1 Introduction

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor that consists of two subunits, HIF-1 α and HIF-1 β . By binding to specific DNA sequence, HIF-1 regulates the transcriptions of several dozen target genes, including vascular endothelial growth factor (VEGF), erythro-

poietin (EPO), and other genes coding for proteins involved in oxygen homeostasis^[1]. Since HIF-1 β can also form heterodimers with other proteins, most research in HIF-1 has been focused on HIF-1 α , which is an oxygen-regulated subunit of HIF-1^[2]. Recent studies indicate that the HIF-1 signaling cascade not only mediates the development of oncogenesis^[3] and the effects of ischemia^[4], but also regulates cardiovascular^[5, 6] and neural development^[7].

HIFs are vital to development. The fact that the deletion of HIF-1 α gene in mammals results in perinatal death makes it hard to investigate *in vivo* the function of HIF-1 α , thus it is difficult to determine the exact role of HIF-1 α in embryogenesis. Instead of gene knockout, small interfering RNA (siRNA) serves as a valuable research tool by inhibiting expressions

Corresponding author: Ze-gui LI, Zhi-jie CHANG

Tel: 023-68753736, 010-62785076

E-mail: lizegui@mail.tmmu.com.cn, zhijie@tsinghua.edu.cn

*Present address: Department of Oncology, General Hospital of Kunming Military Region, Kunming 650000, China

Article ID: 1673-7067(2009)-03-0122-09

CLC number: Q7

Document code: A

Received date: 2008-12-02

of specific genes in mammals. For RNA interference (RNAi), vectors are firstly designed to express short hairpin RNA (shRNA), which is then processed to be a 21nt sequence, similar with the siRNA molecule. After that, RNAi is initiated, with the subsequent post-transcriptional gene silencing (PTGS). By this means, siRNA can be stably expressed in mammalia cells after both transient and stable transfections, and thus the target gene expression can be inhibited continuously and steadily^[8, 9].

Therefore, it is assumed that by transfecting RNA interference plasmid targeting HIF-1 α into the whole embryo *in vitro* using whole embryo culture technique, the role of HIF-1 signaling cascade in neural tube development and dysfunction can be investigated, and observations in embryonic morphological alterations will be greatly facilitated^[10]. The *in vitro* embryonic development is an early state of organogenesis, during which morphological alterations can be observed, including neural tube closure, cardiac development and the limb growth, together with expression changes of some associated downstream genes.

In the present study, specific RNA-interference plasmids targeting mouse HIF-1 α gene were designed and constructed using DNA vectors. Their specificities and efficiencies in inhibiting HIF-1 α expression were also investigated in cell lines. The results will provide a valuable tool to investigate the role of HIF-1 pathway in embryogenesis, oncogenesis and ischemia development in the future.

2 Materials and methods

2.1 Plasmid and strain Plasmids pBS/U6 and pBS/U6/EGFPi were generous gifts from professor Yang Shi in Harvard University, Boston, MA, USA. E.coli DH5 α was stored in

our laboratory.

2.2 Cell lines and main agents 293T and SH-SY5Y cell lines were stored in our laboratory. Restriction enzymes, DNA Ligation Kit Ver.2.1 and One Step RT-PCR Kit were all purchased from Takara, Japan. Other agents for plasmid construction included Klenow Large fragment (Promega, USA), Nucleo Trap DNA Extraction Kit (Clon Tech, USA) and Rapid Plasmid Extraction Kit (Beijing BioDev-Tech. Co., Ltd China). Cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (Gibco Corporation, USA). RNA was isolated using Trizol (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd, China). Biotech hydrophobic polyvinylidene difluoride (PVDF) membrane was from Amersham Pharmacia Biotech, USA. EGFP and antibodies for β -actin, Myc and HA were purchased from Santa Cruz, USA. Fluorescein linked anti-mouse IgG and anti-rabbit IgG, alkaline phosphatase conjugated anti-fluorescein and ECL system were all from Amersham Pharmacia Biotech, USA. Normal goat serum and goat anti-mouse TRITC antibody were from Beijing Zhongshan Golden Bridge Biotech CO., LTD, China.

2.3 Design of siRNA targeting HIF-1 α According to basic principles, the screened sequences were firstly blasted in mouse genome database to ensure the specificity to HIF-1 α gene. Then three specific sequences were selected for designing HIF-1 α siRNA. Six oligonucleotides with restriction sites of ApaI and EcoRI at each side were synthesized using modified construct method, and the targeted sequences of HIF-1 α were 1520-1538, 2349-2367, 2641-2659, respectively (Table 1).

2.4 Construction of interfering plasmid The pBS/U6 vector with a U6 promoter was used to construct a 22 bp siRNA.

Table 1. Sequences of HIF-1 α siRNA oligonucleotides

Site	Sense(5'→3')	Antisense(5'→3')
I(1520-1538)	ATCAACAACCTGAAGATGTTCAAGAGA CATCTTCAAGTTGTTGATCTTTTTTG	AATTCAAAAAAGATCAACAACCTGAAGATG TCTCTGAACATCTTCAAGTTGTTGAT
II(2349-2367)	CCTTAAGCTGCTGCCACTTCAAGAGA GTGGCAGACAGCTTAAGGCTTTTTTG	AATTCAAAAAAGCCTTAAGCTGCTGCCAC TCTCTGAAGTGGCAGACAGCTTAAGG
III(2641-2659)	TCAATGGATGAGAGTGATTCAAGAGA TCCACTCTCATCCATTGACTTTTTTG	AATTCAAAAAAGTCAATGGATGAGAGTGGA TCTCTGAATCCACTCTCATCCATTGA

The hairpin cDNAs were generated through annealing of the complementary oligos synthesized, where ApaI and EcoRI sites were constructed. The hairpin cDNA was subcloned into the pBS/U6 vector at ApaI and EcoRI sites. The clones were verified by KpnI and XhoI digestion and named as siRNA-I, II, III.

2.5 Cell culture and transfection Cells were cultured at 37 °C in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS). 293T cells were plated at a density of 1.5×10^5 per well and cultured at 37 °C with 5% CO₂ overnight for transfection. A total amount of 5 µg DNA per well were transfected into the cells with CalPhos according to the manufacturer's protocol. Then cells were cultured for 36-48 h under normoxia conditions (supplemented with 20% O₂, 5% CO₂ and 75% N₂), followed by hypoxia incubation (supplemented with 1% O₂, 5% CO₂ and 94% N₂) for 6 h. Finally, cells were harvested for later experiments.

2.6 Western blot For protein detection, cells were harvested 48 h post transfection and lysed with 0.3 mL lysis buffer on ice (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100, 1 mmol/L NaVO₃, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerol phosphate, 1 mmol/L phenylmethylsulfonyl fluoride, 5 µg/mL aprotinin, and 5 µg/mL leupeptin, pH 7.5). Total protein (30 µg) was separated by 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and was electro-transferred to PVDF membrane. Membranes were then incubated with antibodies against HIF-1α, enhanced green fluorescent protein (EGFP) and β-actin, respectively, and following steps were carried out according to basic protocols. Finally, the proteins were visualized with enhanced chemiluminescence kit (ECL, Amersham). Assays were repeated three times and the mean value was calculated as the final result. The β-actin protein was used as an internal control. Figures were analyzed by Quantity One 4.4.0 software (BIO-RAD, USA). The protein levels of HIF-1α and EGFP were presented by the

mean relative gray scale (RGS). $RGS = X(\text{Area} \times \text{Density}) / \beta\text{-actin}(\text{Area} \times \text{Density})$, X represents the target gene.

2.7 Immunostaining Transfected 293T cells were cultured under normoxia conditions for 36 h followed by 6-h hypoxia incubation. Cells were washed with PBS for 3 times and fixed in 4% paraformaldehyde for 15 min at room temperature. Then cells were permeabilized by 3% PBS/TritonX-100 (PBST) containing 0.2% Triton X-100 for 10 min. After being blocked with FDB (PBS with 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, 5% normal goat serum, 10% fetal bovine serum and 2% bovine serum albumin) for 30 min, cells were incubated with anti-HA antibody for 2 h, followed by incubation with fluorescent isothiocyanate (TRITC) conjugated goat anti-mouse IgG for 40 min in the darkness. Cells were washed with PBS three times before DAPI treatment. Results were analyzed with Nikon TE300 fluorescence microscope. Pictures were taken with a CCD camera (SPOT, Diagnostic Instrument, USA) equipped with a computer-based image acquisition system.

2.8 RNA isolation and reverse transcriptase-polymerase chain reaction analysis (RT-PCR) Total RNA was extracted using Trizol reagent. Primers were synthesized on the basis of the designed sequences using Primer Premier 5.0 software (Table 2). β-actin was used as an internal control. All the primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. RT-PCR reaction was performed using the One Step RT-PCR Kit (Takara, Japan) with 1 µg of total RNA for each sample. PCR for HIF-1α cDNA was performed at 50 °C for 30 min, 94 °C for 5 min, then repeated 35 cycles at 94 °C for 30 s, 63 °C for 30 s and 72 °C for 3 min. For β-actin, the cDNA was amplified with 25 cycles at 94 °C for 30 s, 55 °C for 50 s, and extension at 68 °C for 1 min. PCR products were separated by electrophoresis using 1% (W/V) agarose gel containing ethidium bromide. Specific bands corresponding to the predicted size were visualized under ultraviolet transilluminator. Assays were repeated 3 times and the mean value was calculated as the final

Table 2. Primers for RT-PCR

Gene	Primer sense(5'→3')	Primer antisense(5'→3')
HIF-1α	ACCGATTCGCCATGGAGG	GTAACTTGATCCAAAGCTCTGAG
β-actin	CAAGAGATGGCCACGGCTGCT	TCCTTCTGCATCCTGTCGGCA

result. Electrophoresis results were analyzed by Quantity One 4.4.0 software (BIO-RAD, USA). The relative amount of HIF-1 α mRNA was calculated from the ratio of HIF-1 α band absorbance to β -actin band absorbance. The inhibition ratio of HIF-1 α mRNA expression was calculated as follows: inhibition ratio = (1 - HIF-1 α mRNA expression level in transfection group / HIF-1 α mRNA expression level in control group) $\times 100\%$.

2.9 Statistical analysis Data were expressed as mean $\pm$ SD and analyzed statistically with one-way ANOVA test using the software package SPSS 11.5. $P < 0.05$ was considered as statistically significant.

3 Results

3.1 Construction of siRNA targeting mouse HIF-1 α gene

Mouse HIF-1 α cDNA (GenBank NM. 010431) was used as a template and the sequences of +1520-1538, +2349-2367, +2641-2659 in HIF-1 α cDNA were selected for RNAi construction, respectively. The enzymatic sites of ApaI and EcoRI were selected for the insertion of a 22bp siRNA, which formed a hairpin structure generated through annealing of the complementary oligos. Clones were verified by KpnI/EcoRI and KpnI/XhoI digestion. As shown in Fig. 1, if interfering vector could be digested by single enzyme, the linearization vector would be generated, which was known as pBlueScript frame (3kb). For KpnI/EcoRI digestion in recombinant clone, the produced fragment containing U6 promoter and interfering sequence (such as the siRNA sequences at sites of +1520-1538 and +2349-2367), would be larger than that from control plasmid pBS/U6. The positive plasmids were named as pBS/U6/HIF-1 α -siRNA-I, II, III (Fig. 1).

3.2 Specific inhibition of HIF-1 α protein level in 293T cells by pBS/U6/HIF-1 α -siRNA The eukaryotic expression plasmid pcDNA3.1-HA-HIF1 α and the interfering plasmid for HIF1 α were co-transfected into 293T cells. Meanwhile, pEGFP and pBS/U6/GFPi were also transiently transfected to detect the specificity of the HIF-1 α interfering plasmid. DNA transfection efficiencies were balanced according to EGFP expression. As shown in Fig. 2, all the three pBS/U6/HIF1 α interfering plasmids could inhibit the expression of HIF-1 α protein in 293T cells to a much lower level (lane 3, 4 and 5),

Fig. 1 Restriction enzyme analysis of pBS/U6/HIF-1 α -siRNA. The enzymatic sites of ApaI and EcoRI were selected for the insertion of a 22bp siRNA. Lane 1: DNA marker; lane 2, 4, 6: KpnI/XhoI digestion of pBS/U6 and pBS/U6/HIF-1 α -siRNA. For plasmids pBS/U6/HIF-1 α -siRNA-I and II, only one large segment cut at KpnI site could be observed since XhoI site was replaced by interfering sequence (lane 4, 6); lane 3, 5, 7: Digest products at KpnI and EcoRI sites of pBS/U6 and pBS/U6/HIF-1 α -siRNA. The fragments from pBS/U6/HIF-1 α -siRNA-I, II were larger than that from pBS/U6 (lane 3). Plasmid pBS/U6 was used as a control.

Fig. 2 Western blot analysis of HIF-1 α and EGFP protein expressions in 293T cells. Lane 1-5 represent the five groups with different transfection treatments. All the three pBS/U6/HIF1 α interfering plasmids could greatly inhibit the protein level of HIF-1 α , among which pBS/U6/HIF-1 α -II exerted the most significant effect. None of the three interfering plasmids showed any interference in EGFP expression, while plasmid U6-GFPi did, indicating the specificities of the three interfering plasmids. β -actin served as a loading control. EGFP was used to detect the specificity of the interfering plasmid.

among which plasmid pBS/U6/HIF-1 α -II (lane 4, interfering at the site of 2349~2367) showed the most significant inhibition, with interfering efficiency of 67.0%. Besides, when compared with plasmid U6-GFPi (lane 2), the three pBS/U6/HIF1 α plasmids exhibited no interfering effect in EGFP expression, which confirmed the specificities of pBS/U6/HIF1 α interfering plasmids. Table 3 lists the RGS of HIF- α protein in 293T cells in 5 groups.

3.3 Immunostaining analysis of inhibition in HIF-1 α protein level by pBS/U6/HIF1 α i-I~III Immunostaining assay

was employed to investigate the silencing effect on HIF-1 α protein expression in transfected cells using anti-HA antibody. Meanwhile, EGFP expression was assessed using fluorescence microscopy. Results showed unaltered expression level of EGFP in cells transfected with both the expression vector and pBS/U6/HIF1 α i-I~III plasmids (Fig. 3g,j and m compared with a) and a much lower level of EGFP in cells transfected with pBS/U6/EGFPi (Fig. 3d), which indicated the specificities of pBS/U6/HIF1 α i-I~III. Moreover, pBS/U6/HIF1 α i-I~III could inhibit HIF-1 α protein level (Fig. 3 h, k and n) when

Fig. 3 Immunostaining analysis of HIF-1 α protein expression in 293T cells. Left row: expression of co-transfected EGFP (Enhanced Green fluorescent protein); middle row: expression of HIF-1 α ; right row: merged images. pBS/U6 served as a control. The cells were immunostained with anti-HA antibody plus TRITC-conjugated secondary antibody (red). EGFP was used to detect the interfering specificity of pBS/U6/HIF1 α i-I~III, and its expression was suppressed by GFPi (indicated as U6-EGFPi). Scale bar: 50 μ m

compared with PBS/U6 or pBS/U6/EGFPi (Fig. 3*b* and *e*), and the interfering effect of pBS/U6/HIF1 α i-II was the strongest among the 3 plasmids, which was consistent with western blot result. These data suggested that pBS/U6/HIF1 α i-I~III could significantly block HIF-1 α protein expression in 293T cells.

3.4 Specific silencing of HIF-1 α mRNA expression in 293T cells by pBS/U6/HIF-1 α - siRNA To investigate the mechanisms underlying the silencing of HIF-1 α protein expression by siRNAs, semi-quantitative RT-PCR was performed. As

shown in Fig. 4, pBS/U6/HIF1 α i-I~III could inhibit the mRNA expression of HIF-1 α (lane 3, 4 and 5) compared with control group (lane 2), while they all exerted no effect on β -actin mRNA level. Among the 3 plasmids, pBS/U6/HIF1 α i-II (interfering at the site of 2349~2367) showed the strongest effect (Table 4), which was consistent with the results of western blot and immunostaining, with an interfering efficiency of 71.3%. These data indicated that pBS/U6/HIF1 α i-I~III inhibited HIF-1 α protein expression through blocking mRNA expression.

Table 3. RGS of HIF-1 α protein expression in 293T cells in 5 groups

Experimental group	1	2	3	4	5
RGS _{HIF-1α}	0.873 $\pm$ 0.017	0.769 $\pm$ 0.024	0.485 $\pm$ 0.015	0.288 $\pm$ 0.011	0.453 $\pm$ 0.021

Fig. 4 Semi-quantitative RT-PCR analysis of HIF-1 α mRNA expression in 293T cells after co-transfection with mouse HIF-1 α expression plasmid and siRNA plasmid. Lane 1: Wild Type 293T, no HIF-1 α amplification product could be detected using specific primers; lane 2: mouse HIF-1 α mRNA was detected in cells transfected with pBS/U6 and pcDNA3.1-HA-HIF1 α ; lane 3, 4, 5: cells were co-transfected with pcDNA3.1-HA-HIF1 α and pBS/U6/HIF-1 α -siRNA-I, II, III, respectively. pBS/U6/HIF1 α i-I~III could inhibit the mRNA expression level of HIF-1 α when compared with control group. β -actin was used as a loading control.

Fig. 5 Western blot analysis of the dose effect of siRNA on the expression of HIF-1 α protein in SH-SY5Y cells. β -actin was used as a loading control. EGFP was used to detect the specificity of pBS/U6/HIF1 α i-II.

Fig. 6 RT-PCR analysis of the inhibition effect of pBS/U6/HIF1 α iII on HIF-1 α mRNA expression in SH-SY5Y cells. Lane 1: pBS/U6; lane 2: pBS/U6-GFPi; lane 3: pBS/U6/HIF-1 α -siRNA-II (0.5 ug); lane 4: pBS/U6/HIF-1 α -siRNA-II (1.0 ug); lane 5: pBS/U6/HIF-1 α -siRNA-II (2.0 ug); lane 6: pBS/U6/HIF-1 α -siRNA-II (3.0 ug). HIF-1 α mRNA expression level was gradually decreased in a dose-dependent manner as indicated in lane 3-6.

Table 4. Relative amount of HIF-1 α mRNA in 293T cells after co-transfection with mouse HIF-1 α expression plasmid and siRNA plasmid

Experimental group	1	2	3	4	5
$A_{\text{HIF-1}\alpha}/A_{\beta\text{-actin}}$	0	1.566 $\pm$ 0.042	1.185 $\pm$ 0.026	0.450 $\pm$ 0.018	0.962 $\pm$ 0.023

Table 5. Western blot analysis revealed a dose effect of siRNA on the expression of HIF-1 α protein in SH-SY5Y cells

Experimental group	1	2	3	4	5	6
$\text{RGS}_{\text{HIF-1}\alpha}$	0.671 $\pm$ 0.022	0.688 $\pm$ 0.030	0.613 $\pm$ 0.027	0.425 $\pm$ 0.018	0.267 $\pm$ 0.014	0.120 $\pm$ 0.002

Table 6. RT-PCR analysis revealed that HIF-1 α mRNA expression level was gradually decreased in a dose-dependent manner

Experimental group	1	2	3	4	5	6
$A_{\text{HIF-1}\alpha}/A_{\beta\text{-actin}}$	1.276 $\pm$ 0.039	1.188 $\pm$ 0.028	0.898 $\pm$ 0.020	0.322 $\pm$ 0.011	0.717 $\pm$ 0.016	0.169 $\pm$ 0.007

3.5 Dose-effect relationship between HIF-1 α protein expression and plasmid pBS/U6/HIF1 α i-II To determine whether some dose-effect relationships exist between the effects of HIF-1 α gene silencing and the dose of plasmid pBS/U6/HIF1 α i, interfering plasmid pBS/U6/HIF1 α i-II was employed for transfection into SH-SY5Y cells with 4 different doses (0.5 μ g, 1.0 μ g, 2.0 μ g and 3.0 μ g), and the plasmid pcDNA3.1-HA-HIF-1 α was also co-transfected to express HIF-1 α . As shown in Fig. 5, results revealed a dose-dependent inhibition in HIF-1 α protein level: with the increase of pBS/U6/HIF1 α i-II dose, the inhibition in HIF-1 α protein expression became stronger (Fig. 5, lane3-6, Table 5). Moreover, the plasmid pBS/U6/HIF1 α i-II had no inhibitory effect on EGFP protein level, while EGFP protein level was greatly reduced in cells co-transfected by EGFP interfering plasmid (lane 2). The inhibitory efficiency of interfering plasmid pBS/U6/HIF1 α i-II at the dose of 3.0 μ g was 82.1%, which was the highest among the four doses, demonstrating the dose-effect relationship between the effect of HIF-1 α gene silencing and the dose of interfering plasmid pBS/U6/HIF1 α i-II.

3.6 Dose-effect relationship between HIF-1 α mRNA expression and the dose of plasmid pBS/U6/HIF1 α i The target gene silencing at mRNA level in SH-SY5Y cells was also observed using semi-quantitative RT-PCR. As shown in Fig. 6, HIF-1 α mRNA expression level was gradually decreased in a dose-dependent manner, data of which were shown in Table

6. At the dose of 3.0 μ g pBS/U6/HIF1 α i-II, the inhibiting rate of HIF-1 α mRNA expression reached up to 86.8%.

4 Discussion

Although it has been widely accepted that the HIF-1 pathway is mainly involved in the regulation of cardiovascular development, the role of HIF-1 in neural development has not been recognized until recently^[12-14]. Our previous studies revealed the expression of HIF-1 α mRNA in developing neural tube, and two characteristics were also identified^[15]. One is that HIF-1 α mRNA is often expressed in locations with high frequency of NTD, such as the anterior neuropore and posterior neuropore at embryonic day 8.5 before they are closed, both of which are vital for normal embryogenesis, since the failure in closing anterior neuropore could result in anencephalia while failure in closing posterior neuropore induces the spina bifida. Another characteristic is that HIF-1 α mRNA is always expressed in locations exhibiting dramatic morphological changes, as well as cellular proliferation and differentiation. For example, HIF-1 α mRNA expression is greatly increased in prosencephalon vesicle which progresses to telencephalon and diencephalon, and in rhombencephalon vesicle from which the pontine brain, cerebellum, and medulla are derived. While in mesencephalon vesicle which would progress towards midbrain formation, there are less morphological alterations, and HIF-1 α mRNA expression is

detected in a much lower level. Above findings suggest that HIF-1 pathway may play an important role in both neural tube closure and normal brain development. However, further research is still required for discovering the mechanism of interference in neural tube closure caused by loss of HIF-1 α expression, which calls for a proper method for investigation.

In previous studies on HIF-1's role in embryogenesis, gene knockout was usually employed to produce HIF1 $\alpha^{-/-}$ mouse model^[18]. However, there are mainly two deficiencies for gene knockout. One is that HIF-1 α knockout may contribute to peripheral dysfunctions, other than the mere interference in target site; another deficiency is the possibility of compensating effect of other genes, which makes it hard to exclude other factors in data analysis. Instead of gene knockout, small interfering RNA (siRNA) serves as a valuable research tool by inhibiting expressions of specific genes with high specificity and efficiency, and has been successfully employed in mammalia studies^[16,17]. An effective siRNA sequence has always been a key determinant for RNAi^[18], since the effectiveness of gene silencing largely depends on target sequence, the latter of which is selected either randomly or through comparing the effectiveness of different target sequences. In the present study, three RNA-interference plasmids, pBS/U6/HIF1 α i-I~III were successfully constructed by inserting the synthesized specific oligonucleotides into expressing vector pBS/U6 with U6 promoter at the sites of Apa I and EcoR I, and were then verified using Kpn I/EcoR I and Kpn I/Xho I digestion. Immunostaining, western blotting and RT-PCR were employed to detect the expression alterations of HIF-1 α after transfections of the three plasmids into 293T cells. Results showed that all the three RNA-interference plasmids exerted significant inhibitory effects in HIF-1 α expression in 293T cell line, among which pBS/U6/HIF1 α i-II (interfering at the site of 2349~2367) exhibited the highest efficiency. pBS/U6/HIF1 α i-II was further selected for transfection into SH-SY5Y cells with different doses, and results showed a dose-dependent inhibition in HIF-1 α expression. At the dose of 3.0 μ g pBS/U6/HIF1 α i-II, HIF-1 α expression was nearly absent in SH-SY5Y cells, which confirmed the specificity and effectiveness of our constructed

pBS/U6/HIF1 α i-II.

There are two basic forms in designing siRNA sequence, that is, interfering plasmid should be specific to the target gene and exhibit no interference in expressions of other genes. Besides, the siRNA sequence should form a stable double-strand structure to ensure effective silencing of target mRNA^[18,19]. Thus, a specific sequence in target gene is critical in siRNA construction. In the present study, the selected sequence was verified to share no homology with other genes, which ensured the specificity in interfering HIF-1 α expression by constructed RNAi plasmid. Besides, since inserted sequences are supposed to form hairpins, the designs of oligonucleotides, pBS/U6 based construction of plasmids, as well as clones of transformant are especially critical. The promoter U6 should still possess RNA polIII activity after the insertions of two oligonucleotides into pBS/U6 vector, which is responsible for the formation of double-strand RNA and silencing the target gene expression. Based on the above principles, we successfully constructed pBS/U6/HIF1 α i-II plasmid interfering at the site of 2349-2347. *In vitro* studies revealed that pBS/U6/HIF1 α i-II could inhibit HIF-1 α expression in both protein and mRNA levels. Results provide a valuable tool in further research discovering the role of HIF-1 α signal cascade in embryogenesis, oncogenesis and other ischemic diseases.

Acknowledgement: This work was supported by the Innovative Foundation of the Third Military Medical University (N0.2007XG42). We thank Dr. Yang Shi in Harvard University, Boston, MA, USA for providing the plasmids pBS/U6 and pBS/U6/EGFPi.

References:

- [1] Semenza GL. Expression of hypoxia-inducible factor 1: mechanisms and consequences. *Biochem Pharmacol* 2000, 59(1): 47-53.
- [2] Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, *et al.* Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science* 2001, 292(5516): 468-472.
- [3] Dang CV, Kim JW, Gao P, Yustein J. The interplay between

- MYC and HIF in cancer. *Nat Rev Cancer* 2008, 8(1): 51-56.
- [4] Yang MH, Wu MZ, Chiou SH, Chen PM, Chang SY, Liu CJ, *et al.* Direct regulation of TWIST by HIF-1 α promotes metastasis. *Nat Cell Biol* 2008, 10(3): 295-305.
- [5] Lee YM, Jeong CH, Koo SY, Son MJ, Song HS, Bae SK, *et al.* Determination of hypoxic region by hypoxia marker in developing mouse embryos *in vivo*: a possible signal for vessel development. *Dev Dyn* 2001, 220(2): 175-186.
- [6] Ylikorkala A, Rossi DJ, Korsisaari N, Luukko K, Alitalo K, Henkemeyer M, *et al.* Vascular abnormalities and deregulation of VEGF in Lkb1-deficient mice. *Science* 2001, 293(5533): 1323-1326.
- [7] Zhu LL, Wu LY, Yew DT, Fan M. Effects of hypoxia on the proliferation and differentiation of NSCs. *Mol Neurobiol* 2005, 31(1-3): 231-242.
- [8] Zhang J, Wang C, Ke N, Bliesath J, Chionis J, He QS, *et al.* A more efficient RNAi inducible system for tight regulation of gene expression in mammalian cells and xenograft animals. *RNA* 2007, 13(8): 1375-1383.
- [9] Scherr M, Eder M. Gene silencing by small regulatory RNAs in mammalian cells. *Cell Cycle* 2007, 6(4): 444-449.
- [10] Calegari F, Haubensak W, Yang D, Huttner WB, Buchholz F. Tissue-specific RNA interference in postimplantation mouse embryos with endoribonuclease-prepared short interfering RNA. *Proc Natl Acad Sci U S A* 2002, 99(22): 14236-14240.
- [11] Ramirez-Bergeron DL, Simon MC. Hypoxia-inducible factor and the development of stem cells of the cardiovascular system. *Stem Cells* 2001, 19(4): 279-286.
- [12] Knabe W, Knerlich F, Washausen S, Kietzmann T, Siren AL, Brunnett G, *et al.* Expression patterns of erythropoietin and its receptor in the developing midbrain. *Anat Embryol (Berl)* 2004, 207(6): 503-512.
- [13] Etchevers HC. Early expression of hypoxia-inducible factor 1 α in the chicken embryo. *Gene Expr Patterns* 2003, 3(1): 49-52.
- [14] Li YX, Ding SJ, Xiao L, Guo W, Zhan Q. Desferoxamine preconditioning protects against cerebral ischemia in rats by inducing expressions of hypoxia inducible factor 1 α and erythropoietin. *Neurosci Bull* 2008, 24(2): 89-95.
- [15] Ding ZY, Li ZG, Xing YZ, Ji H, Ma L. The expression of hypoxia-inducible factor-1 in the neurulation of developing nervous system of mouse. *Chin J of Neuroanatomy* 2005, 21(3): 229-232.
- [16] Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001, 411(6836): 494-498.
- [17] Sverdlov ED. RNA interference—a novel mechanism of gene expression regulation and a novel method for study of their functions. *Bioorg Khim* 2001, 27(3): 237-240.
- [18] Westerhout EM, Berkhout B. A systematic analysis of the effect of target RNA structure on RNA interference. *Nucleic Acids Res* 2007, 35(13): 4322-4330.
- [19] Sui G, Soohoo C, Affar el B, Gay F, Shi Y, Forrester WC. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc Natl Acad Sci USA* 2002, 99(8): 5515-5520.

小鼠 HIF-1 α 基因干扰质粒的构建及其体外抑制效果研究

丁震宇², 李泽桂¹, 星懿展⁴, 纪华⁴, 李红丽⁴, 常智杰³

¹ 第三军医大学药学院生药学教研室, 重庆 400038; ² 沈阳军区总医院肿瘤科, 沈阳 110016; ³ 清华大学医学院基因组学研究所, 北京 100084; ⁴ 第三军医大学基础部组织胚胎学教研室, 重庆 400038

摘要: 目的 构建特异的靶向小鼠 HIF-1 α 基因的 RNA 干扰质粒, 检测其对 HIF-1 α 表达的特异性和有效性。方法 用双酶切方法构建了三组靶向小鼠 HIF-1 α 基因的特异性 RNA 干扰质粒, pBS/U6/HIF-1 α -siRNAI~III。采用 RT-PCR、免疫细胞化学染色和 western blotting 分别检测了转染三组干扰质粒对 293T 细胞内 HIF-1 α 表达的影响。检测了 pBS/U6/HIF1 α i-II 质粒对 SH-SY5Y 细胞中 HIF-1 α 表达的量效干扰效应。结果 三组 RNA 干扰质粒均可显著降低 293T 细胞中 HIF-1 α 的表达, 尤以 pBS/U6/HIF1 α i-II 质粒的干扰效果最佳。验证了 pBS/U6/HIF1 α i-II 质粒对 SH-SY5Y 细胞系中 HIF-1 α 的表达存在剂量效应关系。结论 本实验构建的 pBS/U6/HIF1 α i-II 干扰质粒能有效特异地抑制 HIF-1 α 的表达, 其在神经管发育和神经管缺陷中的作用将被进一步探讨。pBS/U6/HIF1 α i-II 的构建还为探讨 HIF-1 途径在胚胎发育、肿瘤发生和缺血性疾病中的作用提供了有用的研究工具。

关键词: 低氧诱导因子-1 α ; RNA 干扰; pBS/U6/HIF-1 α -siRNA; 小鼠