

CHINESE
MEDICAL SCIENCES
JOURNAL

ORIGINAL ARTICLE

Zinc Finger Protein-activating Transcription Factor Up-regulates
Vascular Endothelial Growth Factor-A Expression *in Vitro*[△]

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Key words: zinc finger protein-activating transcription factor; vascular endothelial growth factor; angiogenesis

Objective To construct the zinc finger protein-activating transcription factor (ZFP-ATF) plasmid and evaluate its efficacy in inducing vascular endothelial growth factor (VEGF) expression in EY.HY926 endothelial cells.

Methods Firstly, we constructed the ZFP-ATF plasmid, then testified the quantity of VEGF protein in EY.HY926 endothelial cells after transfected with ZFP-ATF plasmid by Western blot, finally, we used the RT-PCR to testify whether the ZFP-ATF can stimulate expression of VEGF splice variants.

Results The ZFP-ATF DNA sequences were located the multicloning sites of PVAX1 vector between the site of *Bam*H I and *Xho*I. Western blot result showed VEGF expression in EY.HY926 endothelial cells transfected with ZFP-ATF plasmid was significantly higher than that in cells transfected with VEGF165 (19.95 ± 3.95 vs. 12.15 ± 1.55 $\mu\text{g}/\mu\text{L}$, $P < 0.01$). RT-PCR result showed VEGF-A mRNA expression level induced by ZFP-ATF was high than that induced by VEGF165.

Conclusion ZFP-ATF can up-regulate the VEGF-A expression in comparison with VEGF165, which might have beneficial effects in angiogenesis process.

Chin Med Sci J 2012; 27(3):171-175

THE peripheral artery obstructive disease is common nowadays. Epidemiological research in Beijing showed the incidence rate of peripheral artery obstructive disease in people who were

over the 60 years old is 15.3%.¹ In the diabetic patients being over 50 years, the incidence is as high as 19.47%.² Though the medical treatment can improve symptoms, it can not recanalize the occluded vessel.³ At present, the effective treatment is still revascularization surgery. However, the restenosis would occur again after the angioplasty. Patients who suffer from the diabetes or have no vascular outflow tract can not undergo revascularization surgery.⁴ Due to the limited effectiveness of revascularization surgery, therapeutic angiogenesis, which is a pro-

Received for publication March 11, 2012.

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[△]Supported by the National Natural Science Foundation of China (81270399 and 81100226).

cess of involving the growth and development of new capillaries from a pre-existing vasculature, is proposed by us. We seek to treat the disorders of inadequate tissue perfusion with this therapy.

Vascular endothelial growth factor (VEGF)-A is one of the VEGF families encoding a number of splice variants, which is a prototypic angiogenic growth factor. The functionally inequivalent and non-redundant protein isoforms of VEGF-A,⁵ such as VEGF121, 165, 189, 206, can promote the endothelial cell migration, proliferation, meanwhile, involve in the angiogenesis to increase the blood flow of the ischemic lower extremity.⁶ However, single gene delivery of VEGF for therapeutic angiogenesis in ischemia differs from the normal endogenous VEGF expression process, and researches showed the clinic effects of VEGF on angiogenesis are not satisfactory.^{7,8} The specifically designed zinc finger protein-activating transcription factor (ZFP-ATF) can interact with the unique sites in the genome and thus regulate the target gene expression. In this study, we delivered an engineered ZFP-ATF targeting VEGF genes to regulate the expression of natural array of splice variants, comparing with the single VEGF isoform, which is closer to the physiological process *in vivo* and can evoke a potentially therapeutic effect.⁹

MATERIALS AND METHODS

Cell culture

EY.HY926 endothelial cells (Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences) were maintained in DMEM medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum and antibiotics (GIBCO, CA, USA) at 37°C in a humidified atmosphere with 5% CO₂.

Construction and identification of ZFP-ATF plasmid

To induce up-regulation of endogenous VEGF expression, the coding sequences of three-finger proteins were engineered to bind to accessible regions in the VEGF promoter, which were determined by DNase I hypersensitivity site mapping. The constructed zinc-finger DNA-binding domains were coupled to the VP16 or p65 activation domains from nuclear factor-kappa B (NF- κ B), and nuclear translocation signal from the big T antigen of SV40. In addition, the oligomerization sites were involved in the spatial configuration in the transcription process. They were subcloned into the PVAX1 plasmid (Invitrogen) and expressed under direction of the CMV promoter.¹⁰

The product (5 μ L) was then transferred to competent DH5 bacteria (Invitrogen). The positive recombinants were

selected on a Luria-Bertain (LB) plate with kanamycin. The white bacterial colonies were amplified and plasmids were extracted and purified using the QIAquick DNA reagent kit (Qiagen, Germany).

The colony was further determined with the *EcoR* I restriction enzyme (Invitrogen) and 1% agarose gel electrophoresis. The DNA of the positive recombinants was sequenced. The sequences were determined using BLAST analysis (IMGT).

Plasmid transfection

EY.HY926 endothelial cells were divided into four groups: the ZFP-ATF, VEGF165, and two blank groups, with 6 wells in each group. One day before transfection, cells were plated into the 6-well plates at a concentration of 10⁷ cells per well. Four μ g ZFP-ATF or VEGF165 plasmids (Invitrogen) were transfected into EY.HY926 cells using Lipofectamine2000 (Invitrogen) according to manufacturer's instructions, respectively. The cells in the Blank 1 group were incubated with reduced serum media (250 μ L/well, Opti-MEM, GIBCO-BRL Co. Ltd., USA) and those in the Blank 2 group were cultured with media supplemented with Lipofectamine2000 (10 μ L/well). The medium was replaced 4-6 hours after the transfection.

Western blot

At 48 hours after transfection, the cells were collected. Total cell lysates were prepared and analyzed by Western blot as previously described.¹¹ Briefly, rabbit antibody to VEGF (in 1:1000 dilution, Abcam, Cambridge, UK) and mouse antibody to β -actin (in 1:3000 dilution, Abcam) were used to detect VEGF and β -actin, respectively. After 2 hours of incubation with the first antibody, the membranes were incubated with the IgG antibody linked with horseradish peroxidase (1:3000 dilution, Abcam) for 3 hours. Bands were visualized using ECL kit (Promega, USA) on a Kodak Image station 4000mm Pro System (Kodak, Rochester, NY, USA). The density of the bands was quantified by densitometric analysis.

RT-PCR

TRIzol reagent (Invitrogen) was used to extract the total RNA in cells 48 hours after transfection according to the manufacturer's instructions. Prior to reverse transcription, the total RNA was digested with RNase-free DNase (Invitrogen) and the quality was determined by agarose gel electrophoresis and ultraviolet Spectrophotometer analysis. The cDNA was synthesized from the total RNA template using reverse transcriptase (Invitrogen) and Olig (dT)₁₂₋₁₈ primers (Promega, Germany). Primers for VEGF isoforms

Table 1. Primer sequences used for PCR amplification

Genes	Sequences
VEGF-A	Forward: 5'ACTTTCTGCTGTCTTGGGTG3' Reverse: 5'GGCACACAGGATGGCTTGA3'
VEGF-B	Forward: 5'GTGGTGGTGCCCTTGAAGT3' Reverse: 5'ACGGTGGTGGGGAGTGGCA3'
VEGF-C	Forward: 5'TGATAATGAGTGGAGAAAGAC3' Reverse: 5'TGATTGTTACTGGTTTGGGG3'
VEGF-D	Forward: 5'TCAGCATCCCATCGGTCCA3' Reverse: 5'TCTTTCAGCATTTTCACGGC3'

VEGF: vascular endothelial growth factor

are shown in Table 1. The conditions for PCR amplification were as follows: pre-denaturation at 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 seconds, annealing at 50°C for 2 minutes, extension at 60°C for 60 seconds. PCR products were purified using the QIAquick DNA reagent kit (Qiagen). The fragment length of amplified products was determined using an agarose gel electrophoresis.

Statistical analysis

Microsoft Excel software was used for statistical analysis.

All data were expressed as means±SD. Intra-group difference was determined by student's *t* test. *P*<0.05 was considered to be statistically significant.

RESULTS

The nucleotide and deduced amino acid sequences of the ZFP-ATF are shown in Fig. 1. BLAST analysis showed the ZFP-ATF had 807 bp in length and the total number of amino acids was 268. We found the specific targeted DNA sequences were located the multicloning sites of PVAX1 vector between the site of *Bam*H I and *Xho*I.

Western blot analysis showed ZFP-ATF and VEGF165 induced VEGF expression levels in EY.HY926 endothelial cells were 19.95±3.95 and 12.15±1.55 µg/µL, respectively. VEGF levels of the Blank 1 and Blank 2 groups were 1.72±1.33 and 2.12±1.21 µg/µL, respectively. VEGF expression in EY.HY926 endothelial cells transfected with ZFP-ATF plasmid was significantly higher than that transfected with VEGF165 as well as in cells of two blank groups (all *P*<0.01, Fig. 2).

1	ATGGAATCCAGTACCTGCCAGATACAGACGATCGTCACCGATTGAGGAGAAACGTAAA
1	M E F Q Y L P D T D D R H R I E E K R K
61	AGGACATATGAGACCTTCAAGAGCATCATGAAGAAGAGTCTTTCAGCGGACCCACCGAC
21	R T Y E T F K S I M K K S P F S G P T D
121	CCCCGGCCTCCACCTCGACGATTGCTGTGCCTTCCCGCAGCTCAGCTTCTGTCCCAAG
41	P R P P P R R I A V P S R S S A S V P K
181	CCAGCACCCAGCCCTATCCCTTACGTCATCCCTGAGCACCATCAACTATGATGAGTTT
61	P A P Q P Y P F T S S L S T I N Y D E F
241	CCCACCATGGTGTTCCTTCTGGGCAGATCAGCCAGGCCTCGGCCTTGGCCCCGGCCCT
81	P T M V F P S G Q I S Q A S A L A P A P
301	CCCCAAGTCCTGCCCCAGGCTCCAGCCCCTGCCCCTGCTCCAGCCATGGTATCAGCTCG
101	P Q V L P Q A P A P A P A P A M V S A L
361	GCCCAGGCCCCAGCCCTGTCCAGTCCTAGCCCCAGGCCCTCCTCAGGCTGTGGCCCCA
121	A Q A P A P V P V L A P G P P Q A V A P
421	CCTGCCCAAGCCCAAGGCTGGGGAAGGAACGCTGTGAGAGGCCCTGCTGAGCTG
141	P A P K P T Q A G E G T L S E A L L Q L
481	CAGTTTGATGAAGACCTGGGGGCTTGTGGCAACAGCAGACCCAGCTGTGTTC
161	Q F D D E D L G A L L G N S T D P A V F
541	ACAGACCTGGCATCCGTCGACAACTCCGAGTTTCAGCAGCTGCTGAACAGGGCATACT
181	T D L A S V D N S E F Q Q L L N Q G I P
601	GTGGCCCCCACAACTGAGCCCATGCTGATGGAGTACCCTGAGGCTATAACTCGCCTA
201	V A P H T T E P M L M E Y P E A I T R L
661	GTGACAGGGGCCAGAGGCCGCCGACCCAGCTCCTGCTCACTGGGGGCCCCGGGGCTC
221	V T G A Q R P P D P A P A P L G A P G L
721	CCCAATGGCCTCCTTTCAGGAGATGAAGACTTCTCCTCATTGCGGACATGGACTTCTCA
241	P N G L L S G D E D F S S I A D M D F S
781	GCCCTGCTGAGTCAGATCAGCTCTAA
261	A L L S Q I S S *

Figure 1. Nucleotide and amino acid sequences of the zinc finger protein-activating transcription factor (ZFP-ATF).

RT-PCR revealed ZFP-ATF only induced VEGF-A mRNA expression, and there was no difference in mRNA expression levels of VEGF-B, VEGF-C, and VEGF-D between ZFP-ATF and VEGF165 transfected EY.HY926 cells (Fig. 3).

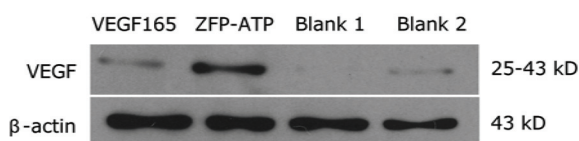


Figure 2. Western blot analysis result of VEGF expression in EY.HY926 endothelial cells after 48 hours of transfection with the designed ZFP-ATF binding to the targeted sequences in the VEGF promoter. β -actin was the loading control.

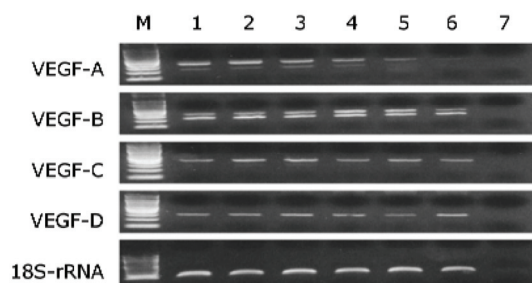


Figure 3. RT-PCR analysis result of VEGF mRNA isoform expression in EY.HY926 endothelial cells after transfection of ZFP-ATF or VEGF165.

M: marker; lanes 1-3: ZFP-ATF transfected EY.HY926 cells; lanes 4-6: VEGF165 transfected EY.HY926 cells; lane 7: EY.HY926 cells cultured with medium alone.

DISCUSSION

Angiogenesis, firstly proposed by Folkman, includes two distinctly main mechanisms: vasculogenesis and angiogenesis.¹² The angiogenesis we studied is stimulated by hypoxia, ischemia, and inflammation, finally, producing the vascular wall by the differentiation and migration of vascular endothelial cell. At present, a variety of angiogenesis-related growth factors have been identified, such as VEGF, fibroblast growth factor, and hepatocyte growth factor, which can respectively stimulate the angiogenesis and treat ischemic disease. However, the regional angiogenesis with VEGF (RAVE) trial carried out by Rajagopalan *et al*¹³ showed that VEGF121 or VEGF165 alone can not effectively reduce the rate of amputation and improve patients' exercise capacity.

In this research, we constructed a novel transcription factor expression plasmid, namely ZFP-ATF, which was mutated at key positions in a given zinc-finger framework,

making the deduced proteins specifically bind to VEGF promoter to up-regulate endogenous VEGF expression.¹⁴⁻¹⁶ Because ZFP-ATF composed of human zinc fingers is less likely to induce a host immune response than mutated ZFP would be,¹⁷ thus, it might be preferable in therapeutic applications. The constructed ZFP-ATF plasmid containing ZFP moieties and an activation domain can encode VEGF expression with great efficiency *in vitro*.

Western blot results showed that the ZFP-ATF group, in contrast to the VEGF165 group, had a more powerful effect to promote VEGF protein expression. And RT-PCR results suggested that ZFP-ATF alone stimulated the VEGF-A expression and simultaneously increased the main VEGF splice variants expression. Though VEGF165 can stimulate the splice variants expression of VEGF, the effect was weak compared with the ZFP-ATF group. Our results demonstrated that the process of ZFP-ATF stimulating VEGF gene expression is closer to the normal physiological procedure in body. ZFP-ATF can meet the requirements for the angiogenesis which needs enough VEGF protein concentration and the major VEGF splice variants.

Taken together, these data further demonstrated that the ZFP-ATF plasmid can specifically interact with VEGF gene promoter, and increase VEGF expression, ultimately leading to the angiogenesis. In gene therapy applications, there are at least three major objectives which are of relevance to the use of artificial transcription factors: maintaining a high level of DNA-binding specificity, achieving a desired level of gene expression in a target cell type and regulating the activity of the targeted gene, moreover, avoiding alteration in the expression of other genes.¹⁸⁻²⁰ Therefore, in addition to the VEGF gene, it is also feasible to design other kinds of ZFP for combination with different DNA sequences, which eventually will regulate the expression of other proteins in need.^{21, 22}

We have proven the ZFP-ATF might be effective in stimulating VEGF-A expression *in vitro*. ZFP-ATF will become an effective and powerful factor in angiogenesis. However, the limitation of the ZFP-ATF is that it can only act on the VEGF gene. Because other factors are involved in the angiogenesis, such as fibroblast growth factor and nerve growth factor,²³ etc. Therefore, whether the ZFP-ATF combining with other growth factors will be more effective and practical in the treatment of ischemic disease deserves to be investigated in the future.

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