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Full Length Research Paper

Synthesis of the human $VEGF_{165}$ gene based on overlap PCR and recombinant expression in stable transfected CHO cells

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Vascular endothelial growth factors (VEGFs) are a member of a family of structurally related proteins that mediate angiogenesis, and vascular maintenance. $VEGF_{165}$ is an isoform and has been studied extensively because of its potential use in therapeutic angiogenesis. In this study, we synthesized the human $VEGF_{165}$ ($hVEGF_{165}$) gene based on overlap PCR method and recombinant expressed in Chinese's hamster ovary (CHO) cell. The coding sequence of $hVEGF_{165}$ was synthesized successfully and the amounts of the recombinant protein could reach to 603.085 pg/ml. This $rhVEGF_{165}$ protein could promote angiogenesis in chicken embryos. In conclusion, we established the stable expression system of recombinant human $VEGF_{165}$ ($rhVEGF_{165}$) protein in CHO cells.

Key words: Overlap extension PCR (OE-PCR), vascular endothelial growth factor (VEGF), Chinese's hamster ovary (CHO) cell, recombinant protein.

INTRODUCTION

Vascular endothelial growth factors (VEGFs) are composed of two identical polypeptide chains; formed homodimer glycoprotein by disulfide bonds, which belong to a member of a structurally related proteins family that acts as ligands for the VEGF receptors' family. VEGF can promote endothelial cell mitosis and angiogenesis (Zachary, 2001) by binding and activating VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2), two structurally related membrane receptor tyrosine kinases, which are mainly expressed by endothelial cells in the blood vessel wall (Ferrara, 2004; Robinson and Stringer, 2001; Tao et al., 2006). The binding of the VEGF to its receptors commences on a signaling cascade that ultimately simulates vascular endothelial cells growth, survival and proliferation, and promotes angiogenesis.

So far, seven glycoproteins, including VEGF-A, VEGF-B, VEGF-C and VEGF-D, VEGF-E, VEGF-F and the placental growth factor (PIGF), have been identified in the VEGF family (Yamazaki and Morita, 2006). Multiple VEGF-A protein isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$, etc.) have also been found, which were formed by alternative splicing of exons (Ferrara et al., 2003). $VEGF_{165}$ shows the strong mitogenic potency to vascular endothelial cells, both in physiological or pathological conditions (Keck et al., 1989; Leung et al., 1989), and confers a strong induced effect on the vascular formation *in vitro* (Kowanetz and Ferrara, 2006). In addition, $VEGF_{165}$ also plays an important role in the clinical gene therapy of promoting angiogenesis in transplanted skin (Zheng et al., 1998) and wound healing (Ferraro et al., 2009).

Recent studies have demonstrated that $rhVEGF_{165}$ protein could be expressed in prokaryotic *Escherichia coli* (Siemeister et al., 1996; Celec et al., 2005; Lee et al., 2010), yeast (Mohanraj et al., 1995; Ma, et al., 2001) and insect cells (Lee et al., 2006). However, the $rhVEGF_{165}$ protein in these systems revealed to be different forms derived from mammal host cell (Grabenhorst et al., 1999), especially in glycosylation respects. The aim

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Abbreviations: VEGFs, Vascular endothelial growth factors; **CHO,** Chinese's hamester ovary; **hVEGF,** human vascular endothelial growth factor; **rhVEGF,** recombinant human vascular endothelial growth factor; **PLGF,** placental growth factor.

Table 1. The sequence of designed oligonucleotides.

Primer	Sequence（5′→ 3′）
P1	GCAAGGCTCCAATGCACCCAAGACAGCAGAAAGTTCAT
P2	GGGTGCATTGGAGCCTTGCCTTGCTGCTCTACCTCCACCATGCCAAGTGGTCCCAGGCT
P3	CTTCGTGATGATTCTGCCCTCCTCCTTCTGCCATGGGTGCAGCCTGGGACCACTTGGCA
P4	GGGCAGAATCATCACGAAGTGGTGAAGTTCATGGATGTCTATCAGCGCAGCTACTGCCA
P5	AGGGTACTCCTGGAAGATGTCCACCAGGGTCTCGATTGGATGGCAGTAGCTGCGCTGAT
P6	CATCTTCCAGGAGTACCCTGATGAGATCGAGTACATCTTCAAGCCATCCTGTGTGCCCC
P7	TCCAGGCCCTCGTCATTGGAGCAGCCCCCGCATCGCATCAGGGGCACACAGGATGGCTT
P8	CCAATGACGAGGGCCTGGAGTGTGTGCCCACTGAGGAGTCCAACATCACCATGCAGATT
P9	TCTCTCCTATGTGCTGGCCTTGGTGAGGTTTGATCCGCATAATCTGCATGGTGATGTTG
P10	GGCCAGCACATAGGAGAGATGAGCTTCCTACAGCACAACAAATGTGAATGCAGACCAAA
P11	GCAAGGCCCACAGGGATTTTCTTGTCTTGCTCTATCTTTCTTTGGTCTGCATTCACATT
P12	AAATCCCTGTGGGCCTTGCTCAGAGCGGAGAAAGCATTTGTTTGTACAAGATCCGCAGA
P13	TTGCAACGCGAGTGTGTGTTTTTGCAGGAACATTTACACGTCTGCGGATCTTGTACAAA
P14	ACACACACTCGCGTTGCAAGGCGAGGCAGCTTGAGTTAAACGAACGTACTTGCAGATGT
P15	TCACCGCCTCGGCTTGTCACATCTGCAAGTACGTTCG

of this study was to obtain the stably expressing $rhVEGF_{165}$ therapeutic protein and to establish a basis for the further research on clinical gene therapy of VEGF.

MATERIALS AND METHODS

Gene synthesis

Fifteen single-strand oligonucleotides were designed according to the coding region of human VEGF gene (GenBank no. AF486837.1) and synthesized by Sangon Biotech (Shanghai) Co., Ltd (Table 1). The sequence of the fifteen primers were reverse complement at the 3'-end between two proximity primers (Liu et al., 2008; Mehrnejad et al., 2008), which interacted as templates for each other to produce the template of the next PCR (Figure 1A).

The four single-stranded oligonucleotide primers, P6, P7, P8 and P9, were selected in the first round of PCR. The obtained PCR fragment was recovered through agarose Gel DNA Fragment Recovery Kit (TaKaRa, Japan). Each round of the PCR reaction products acted as the template of the next round. The target gene was extended by adding the two oligonuleotides primers on each side in turn.

The first part of the gene was amplified by OE-PCR with the oligos P6, P7, P8 and P9 as interactive templates. The PCR program consisted of a denaturation step at 95℃ for 3 min, then 25 cycles at 94℃ for 30 s, 50℃ for 40 s, 72℃ for 30 s and a final extension cycle at 72℃ for 3 min. PCR product was analyzed on 1.5% agarose gel and recovered as described earlier.

The second PCR used P4, P5 and P9 oligos as primers and the first PCR product as the template. Similarly, the third, fourth, fifth, sixth and seventh products could be obtained with the previous PCR product for template and two oligos introduced at one end for primers (Figure 1B). The PCR reaction condition was the same as that of the first PCR.

The final PCR product was purified from agarose gel and cloned into pMD 19-T vector (TaKaRa, Japan) to produce the plasmid pMDV. Three recombinant plasmids were picked up and sequenced by Songon Biotech (Shanghai) Co., Ltd and the sequencing homology was analyzed by Blast.

Plasmids construction

pMDV plasmid was digested by *Hin*d *III* and *Xba* I (TaKaRa, Japan) and inserted into the pCAG vector (Wang et al., 2009) digested with the corresponding enzymes, replacing the CAT gene with $VEGF_{165}$ gene, to generate the resultant plasmid pCGV. The constructs were shown schematically in Figure 2A.

The pCGV vector containing $hVEGF_{165}$ gene was transfected into the competent *Escherichia coli* JM109 cells kept in our laboratory and selected under ampicillin antibiotics (Solarbio, China). The positive clones were picked and cultured, and the pCAG and pCGV plasmids were extracted with Endotoxin-free Plasmid Mini Kit (Solarbio, China) according to the manufacturer's instruction.

Cell culture and transfection

CHO cells were cultured in DMEM (Gibco, US) supplemented with 10% fetal bovine serum (FBS) (Gibco, US), 100 U/ml penicillin-streptomycin solution (Solarbio, China), and 1% L-glutamine (Solarbio, China).

At 24 h prior to the transfection, 8×10^4 to 2×10^5 cells were seeded into a 24 well plate. 0.6 μg of the plasmids were transfected into the cells using the Suohua-Sofast^{TM} transfection reagent (Xiamen Sunma Biotech, China) according to the manufacturer's instructions. Each group were performed in triplicate and allowed to incubate subsequently at 37℃ and 5% CO_2 for 3 h. Thereafter, the complex was removed and replaced with 1 ml of complete medium. After 48 h of gene delivery, transfected cells were selected in medium containing G418 (400 μg/ml) (Songon, Shanghai, China). Stable transfected positive clones appeared after 14 days when the CHO cells not tranfected by plasmid died completely under the same selective pressure. Subsequently, the concentration of G418 in the medium decreased to 200 μg/ml and kept for two weeks. When the number of single colonies grew up to 2×10^5 cells, the free serum

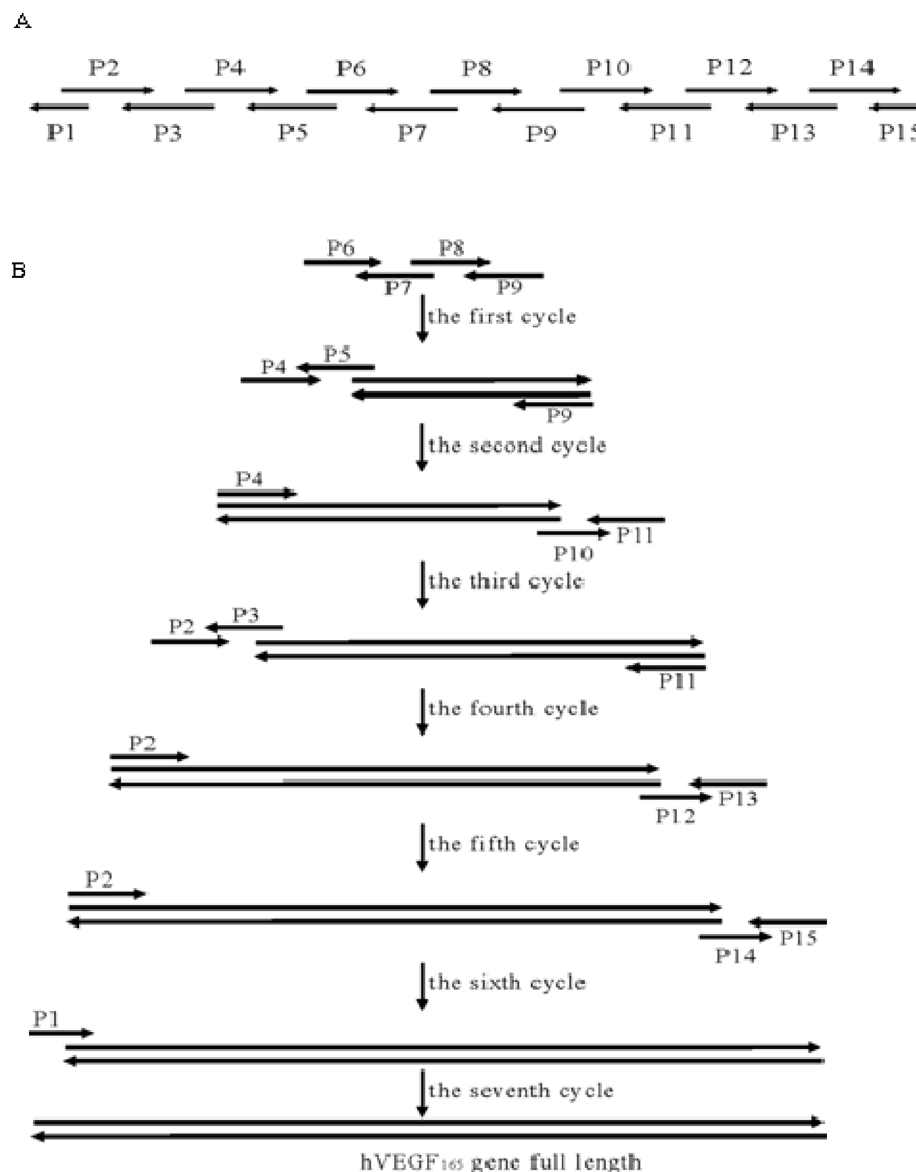

Figure 1. Diagram of $hVEGF_{165}$ synthesis. A: The designed fifteen oligonucleotides; B: The first PCR production was synthesized by P6, P7, P8 and P9 primers, and then acted as the template of the next cycle. Similarly, the previous PCR production acted as the template of the next PCR cycle and introducing two oligos in one ends. The full length $hVEGF_{165}$ gene was ultimately synthesized by seven PCR cycles.

medium was added, and then, the supernatant was collected after 48 h.

Analysis of VEGF expression

VEGF expression was detected by hVEGF ELISA Kit (Adlitteram Diagnostic Laboratories, US) according to the kit instruction. Briefly, each sample (200 µl) was added into the designated well. Series diluted hVEGF standards samples, including 0, 50, 100, 250, 500, 1000 pg/ml, were added to the microwell plates. The mixture was detected by enzyme-linked immunosorbent assay (ELISA) reader (Bio-tek ELx800 microplate reader, US) at 450 nm wavelength 5 min later. The $VEGF_{165}$ concentration in samples was calculated based on a standard curve.

CAM angiogenesis assay

Fertilized chicken eggs were cultured for 7 days under constant humidity at 37°C. A square window (1 cm^2) was opened in the shell and a sterilized filter paper (5 mm^2) that contained the $rhVEGF_{165}$ proteins or physiological saline, was placed on the surface of the CAM. The window was sealed with sterile adhesive tape and the eggs were continued to culture for 72 h and the windows were extended carefully and then photographed.

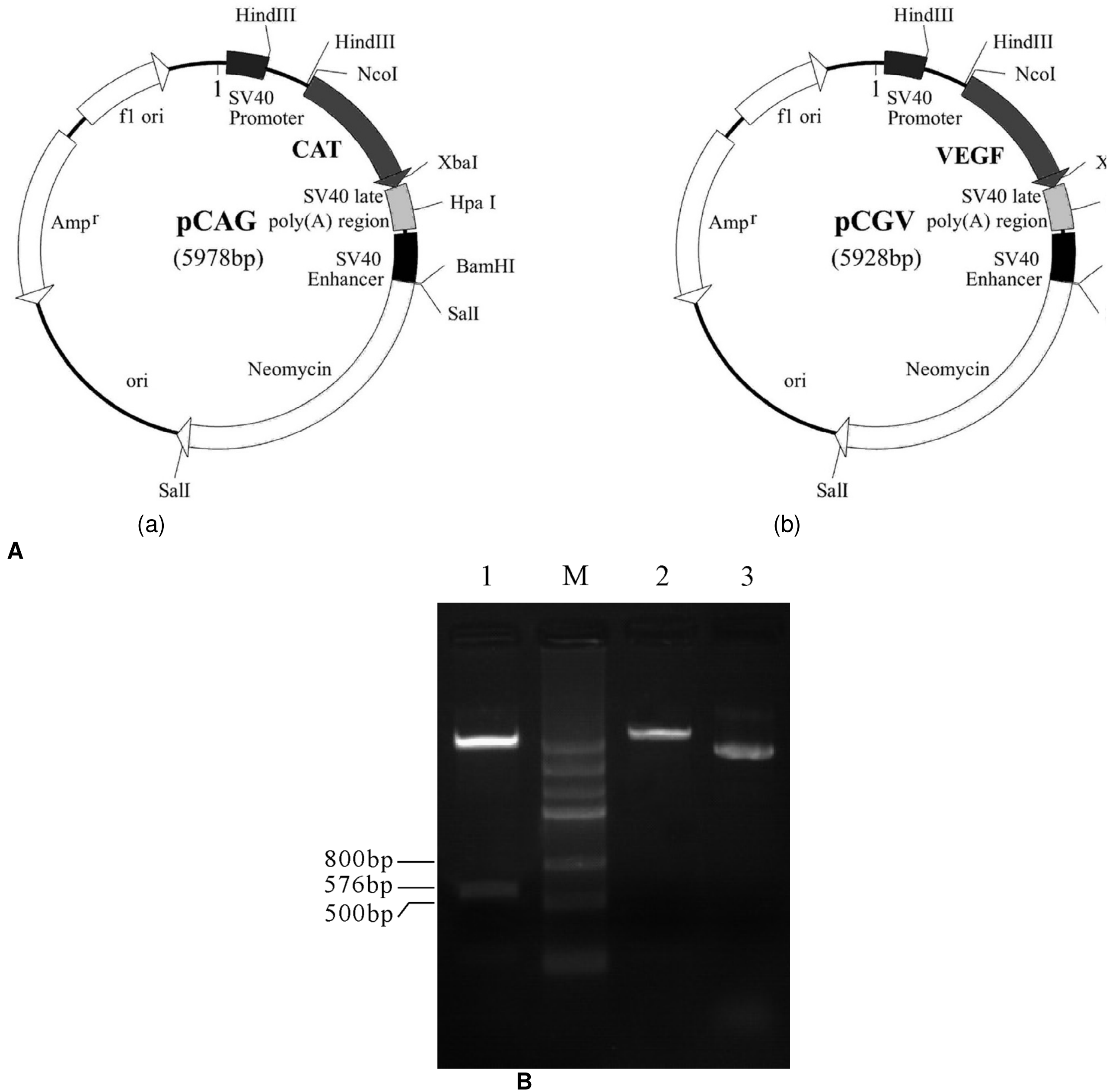

Figure 2. Schematic of vector construction and screening of the recombinant plasmid pCGV. A: (i) pCAG control vector; (ii) pCGV vector: CAT report gene was replaced by hVEGF$_{165}$ gene. B: Lane 1: pCGV plasmid digested by *Hin*d III and *Xba I* (about 5.2 kb + 576 bp); Lane M: DNA Marker III; Lane 2: pCGV plasmid digested by *Hin*d III (about 5.7 kb); Lane 3: pCGV plasmid.

RESULTS AND DISCUSSION

Synthesis of VEGF$_{165}$ gene by OE-PCR

After seven PCR reactions, the results showed that the size of amplified band was consistent with the expected fragment (Figure 3). However, primer-dimers were also noticeable because of the complementarity between the primers.

The final PCR product was cloned into the pMD19-T vector. Clones were selected randomly and digested with *Bam* HI and *Hin*d *III.* Two DNA fragments of approximately 2600 and 576 bp appeared. The sequence of one of the samples was 100% homology with VEGF$_{165}$. A mutation at the point of the 59th nt of P3 primer: “A” mutate to “G” was found in another clone (Figure 4). These findings indicated that the OE-PCR technology could synthesize the interesting genes (Zhang et al., 2005; Cherry et al., 2008; Mehrnejad et al., 2008). In addition, it can also be used to increase the protein

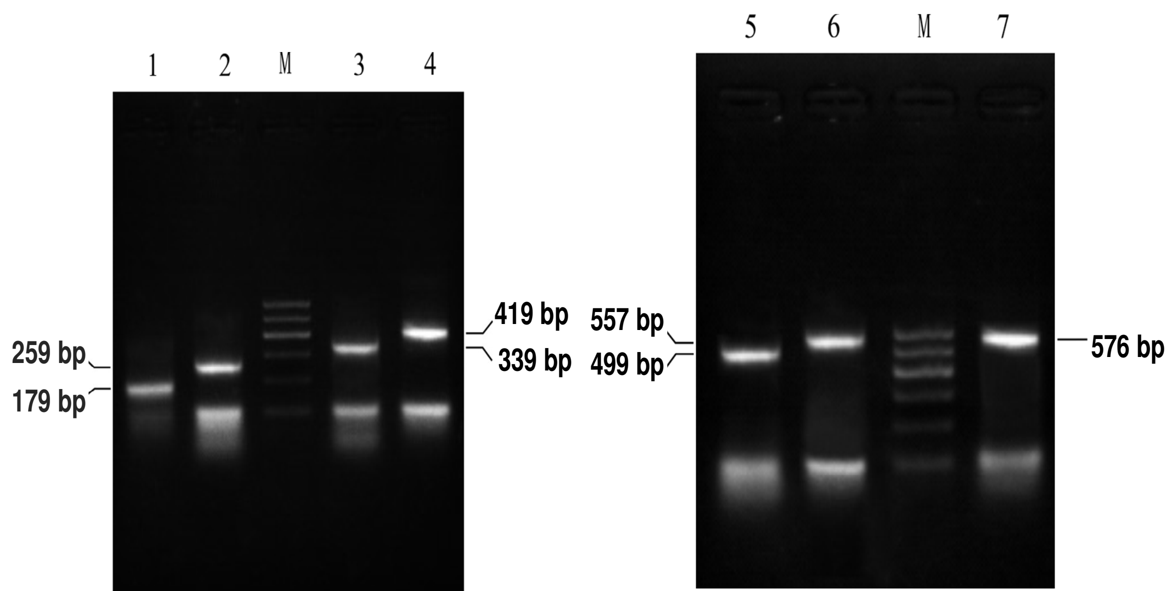

Figure 3. PCR products of seven cycles in synthesizing hVEGF$_{165}$ gene and sequencing of recombinant plasmid pMDV. Lane 1: The PCR product of the first cycle with the primers P6, P7, P8 and P9; Lane 2: The second PCR product with P4, P5 and P9 as primers; Lane 3: Marker I; Land 4: The third PCR production with P4, P10 and P11 as primers; Lane 5: The forth PCR production with P2, P3 and P11 as primers; Lane 6: The fifth PCR product with P2, P12 and P13 as primers; Lane 7: the sixth PCR production with P2, P14 and P15 as primers; Lane 8: Marker I; Lane 9: the seventh PCR product with P1 as primer. The previous PCR product was acted as the PCR template except for the first PCR produce.

production through codon optimization that is genetically modified (Gordeeva et al., 2010).

Construction and identification of plasmid vector

The recombinant plasmid pCGV was digested by *Hin*d III and *Xba* I. The size of two digested DNA fragment were approximately 5.2 kb and 576 bp (Figure 2B), which was consisted with the pCAG vector that CAT report gene was replaced by hVEGF$_{165}$ gene. The result demonstrated that the target vector was constructed successfully.

Expression of recombinant protein in CHO cells

The recombinant plasmids (pCAG and pCGV) were stably transfected into CHO cells, respectively. The supernatant medium was collected to evaluate the recombinant VEGF$_{165}$ protein using ELISA. The highest value reached 603.085 pg by 2×10^5 cells, while the lowest was only 369.46 pg under the same conditions (Figure 5B). The expression of recombinant protein is closely connected with the integration sites of foreign genes in the host chromosomes, so the difference of rhVEGF$_{165}$ protein among clones seems to be extremely due to the exogenous gene inserted into the host chromosome sites.

The average protein concentration of the clones transfected with pCGV was 472.64 pg, which was significantly increased than that of the cells transfected with the control pCAG plasmid (Figure 5A), indicating that the rhVEGF$_{165}$ protein could be expressed in the CHO cell.

Angiogenesis effection of rhVEGF$_{165}$ protein

To determine whether rhVEGF$_{165}$ protein could promote new blood vessel growth *in vivo*, the CAM analysis was performed. The result showed that treatment of CAM with the recombinant protein resulted in a more effective angiogenesis effect than that of the 0.65% physiological saline and blank control (Figure 6), which suggested that the rhVEGF$_{165}$ protein has the biological activity in promoting CAM angiogenesis.

VEGF plays a pivotal role in physiological and pathological regulation of angiogenesis. Several studies have indicated the possibility of VEGF in the therapy of ischemic heart disease and cancer. However, especially VEGF gene therapy is associated with the product of hVEGF protein. In this study, we have correctly synthesized the hVEGF coding sequence by OE-PCR, which provide a time-saving, economic method for researchers to amplify the desired full-length sequencing. Two oligonucleotides were introduced in each round of PCR assembly, which can greatly improve the accuracy, speed and efficiency of the gene synthesis.

Figure 4. The sequence of $hVEGF_{165}$ gene synthesized by OE-PCR.

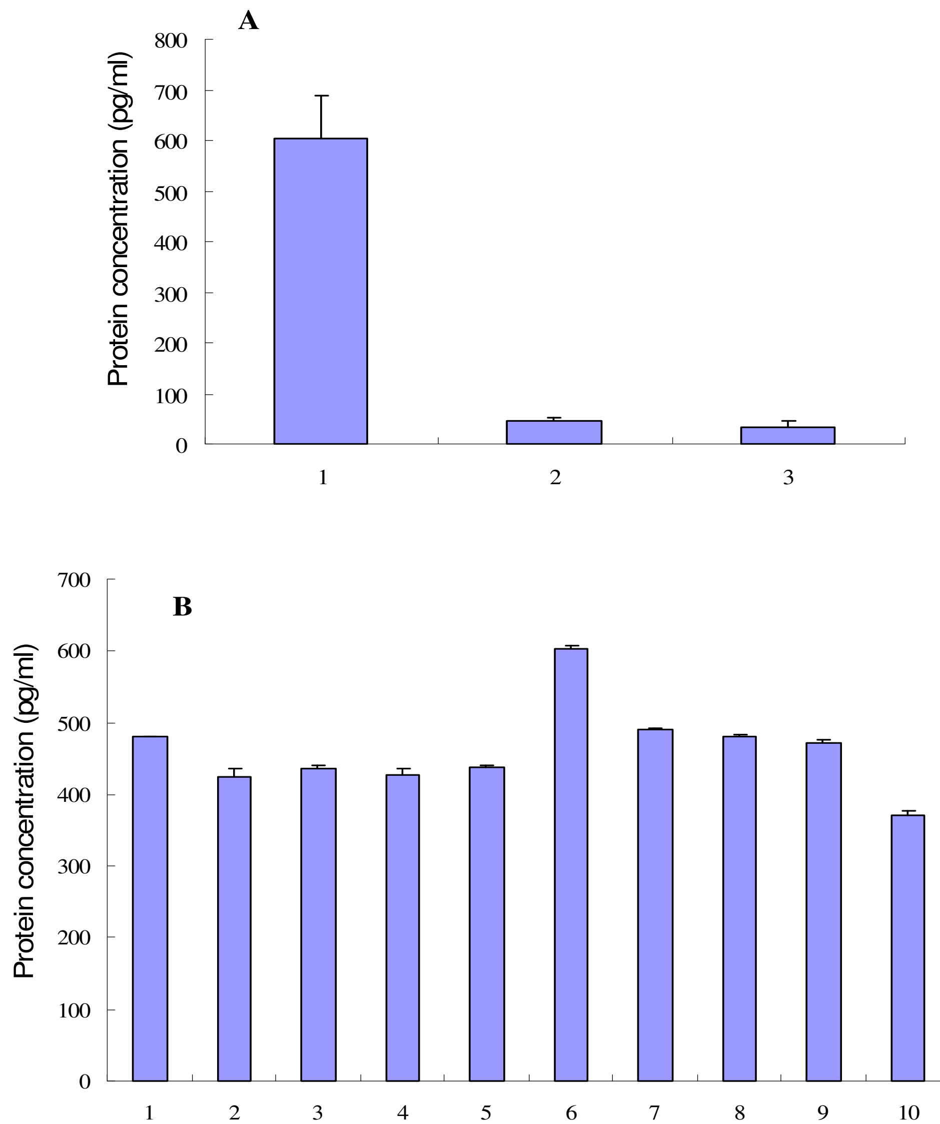

Figure 5. The amount of the $rhVEGF_{165}$ expression in stable transfected CHO cells. A; column represents the amount of $rhVEGF_{165}$ expression. B; the amount of $rhVEGF_{165}$ protein of ten individual clones expressed in the CHO cells transfected with the plasmid pCGV.

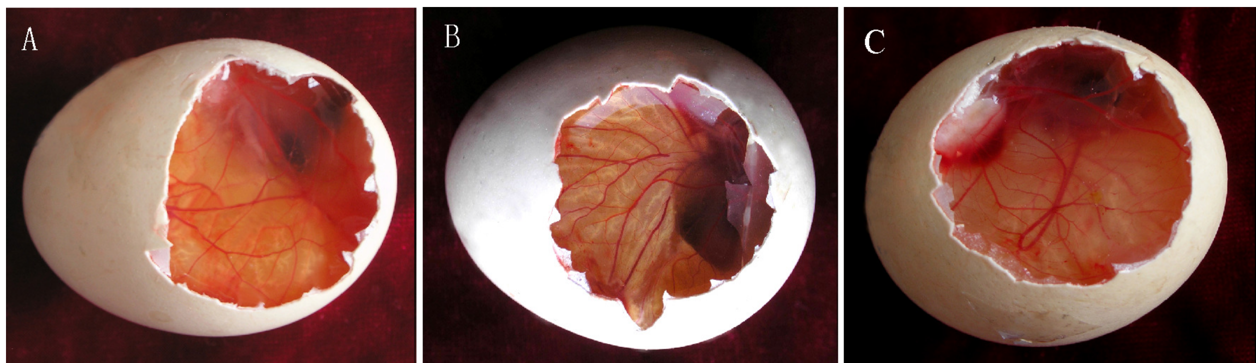

Figure 6. The $rhVEGF_{165}$ protein promotes angiogenesis. A; blank control, opening window with no treatment; B; physiological saline control; C; $rhVEGF_{165}$ protein.

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