Enhanced Expression of Bioactive Recombinant VEGF-111 with Insertion of Intronic Sequence in Mammalian Cell Lines

Zohreh Hojati · Fariba Dehghanian

Abstract Among all VEGF-A isoforms, VEGF-111 is particularly important in molecular biology research owing to its potent angiogenic properties and its remarkable resistance to proteolysis. These features make it a potential candidate for therapeutic use in ischemic diseases. VEGF-111 is not expressed in normal cells, but expression is induced by UV-B irradiation and exposure to genotoxic agents. Here, to increase expression at the transcriptional and translational levels, we synthesized and cloned recombinant VEGF-111 cDNA. Two fragments encoding exons 1–4 and intron 4/5 plus exon 8a were amplified and cloned into the pBud.CE4.1 vector using a class IIs restriction enzyme-based method. The expression of VEGF-111 in CHO-*dhfr* − and HEK 293 cell lines was evaluated with real-time PCR, dot blotting, and ELISA. VEGF expression was increased about 10- and 18-fold in transfected CHO-*dhfr* − and HEK 293 cells, respectively. Dot blotting and ELISA confirmed successful production of VEGF-111 in both cell lines.

Keywords VEGF-111 · Real-time PCR · Dot blotting · ELISA · Intron 4/5

Introduction

Angiogenesis is mediated by a complex regulatory system and is a key component of various physiological and pathological processes in humans [1]. Vascular endothelial growth factor (VEGF) is an interesting angiogenic factor that plays various roles in vascular function and structure [2]. VEGF-A (generally called VEGF), the most potent member of the family, was the first to be discovered and remains the most studied VEGF. VEGF is involved in many pathological conditions that involve excessive or deficient VEGF expression. The coding region of human *VEGF*, which is located on chromosome 6p21.3, spans ~14 kb and contains eight exons. Alternative mRNA splicing and formation of multiple isoforms provide more

Z. Hojati (✉) · F. Dehghanian
Division of Genetics, Biology Department, Faculty of Sciences, University of Isfahan, Isfahan, Iran
e-mail: z.hojati@sci.ui.ac.ir

F. Dehghanian
e-mail: f.dehghanian68@gmail.com

complexity to VEGF biology [3, 4]. Two principal VEGF isoform subfamilies exist, including the proangiogenic VEGF-xxx (VEGF-xxxa) and antiangiogenic VEGF-xxxb isoforms [5, 6].

VEGF-111 was the most recent VEGF isoform to be identified and was discovered by Mineur et al. in several types of cultured cells following treatment with UV-B irradiation and genotoxic drugs. This new isoform of VEGF (encoded by exons 1–4 and 8a) has unique functions and features. The potent angiogenic activity, remarkable resistance to proteolysis, and additional stability for a freely diffusible molecule make VEGF-111 a potential therapeutic agent [7–9]. The absence of exon 5, which contains a proteolytic cleavage site (Arg110–Ala111), makes VEGF-111 more stable by conferring resistance to proteolytic degradation. Exons 6 and 7 encode a basic heparin-binding region, and thus, the absence of this region in VEGF-111 makes it freely diffusible [10, 11]. VEGF-111 mRNA is not expressed in normal conditions in healthy human or murine tissues but is produced in response to genotoxic stress including UV-B and genotoxic factors such as camptothecin, mimosin, and mitomycin C that induce double-stranded breaks [9]. Chemotherapeutic drugs used in cancer treatment induce VEGF-111 expression as an important and stable proangiogenic growth factor that leads to tumor development and metastasis. In this regard, VEGF-111 is an undesired by-product of cancer treatment and a proposed cause of resistance to such treatment. On the other hand, VEGF-111 is an efficient therapeutic tool in pathological situations including myocardial and peripheral ischemic disease, neurodegenerative pathology, healing of chronic wounds, and tendon lesions, the last of which are frequently seen in sports injuries. A recent study demonstrated that VEGF-111 improves the viability of the ovarian cortex by limiting ischemia [12].

Therefore, the synthesis and cloning of recombinant VEGF-111 cDNA represent a promising approach to improve our knowledge about the structure, function, and regulatory processes of this VEGF, especially in cancer development. In addition, cloning of VEGF-111 cDNA will result in a practical tool for use in angiogenesis molecular research, tissue engineering, stem cell therapy, and treatment of ischemic diseases and chronic wounds. Recent studies demonstrate that nuclear processing of pre-mRNA molecules often improves the production yields of recombinant cDNAs. Introns enhance the efficiency of transcription, mRNA nuclear export, and mRNA stability owing to interactions with different types of molecular machinery [13, 14]. Therefore, the purpose of this study was to improve the level of VEGF-111 production in mammalian cell lines by inserting a homologous intronic sequence between exons 4 and 8a.

Furthermore, we used an efficient, accurate, and cost-effective method based on class IIs restriction enzymes to synthesize VEGF-111 recombinant cDNA. The synthesized VEGF-111 recombinant cDNA was cloned into the pBud.CE4.1 vector and transfected into HEK 293 and CHO-*dhfr* − cell lines to analyze VEGF-111 mRNA and protein expression [15, 16].

Materials and Methods

RNA Isolation and cDNA Synthesis from Breast Tumor Tissue

A 20-mg fresh malignant breast tumor tissue was used here for RNA isolation. Fresh tumor tissue sample was collected from the Breast Cancer Research Center of Isfahan. This study was approved by the institutional ethics committee of the University of Isfahan. Patient was informed clearly and provided standardized written consent. For disruption of the tumor tissue, a mortar and pestle along with liquid nitrogen were used. Homogenization was carried out by a syringe and needle. The whole RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and stored in RNase-free water at −80 °C. The quantity and quality of RNA

were measured by spectrophotometer and electrophoresis in a 1 % agarose gel, respectively. Finally, the cDNA was created using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Germany) via Random Hexamer Primers and stored at −80 °C.

Synthesis of the Recombinant VEGF-111 cDNA

The coding sequence of VEGF-111 isoform is composed of exons 1–4 and 8a. To synthesize a recombinant cDNA of the VEGF-111 containing an intronic sequence, two parallel PCRs were designed and performed. In the first reaction, exons 1–4 were amplified using the cDNA of malignant breast tumor tissue. The cells in this tissue produce high levels of *VEGF-A* mRNA, enough for PCR amplification and cloning [17, 18]. The second PCR was designed to amplify the intronic sequence from the chromosomal DNA isolated from the human blood cells (Fig. 1). Blood collection was performed from a healthy free volunteer who had referred to clinics of the Masih Daneshvari Hospital for a regular health checks. Blood sampling was done based on patient satisfaction, and an agreement was signed between the University of Isfahan and the Masih Daneshvari Hospital.

PCR Amplification

Two set primers were designed using the primer design software Oligo 7 for amplification of two fragments which form VEGF-111 recombinant cDNA (Table 1). The recognition site of *Eco*31I enzyme (a class IIs restriction endonuclease) was incorporated into the primers R1 and F2. *Eco*31I cuts four nucleotides (NNNN) outside its recognition site and forms a 4-base 5′ overhang; the base composition of these overhangs was specifically designed for attachment of the amplified fragments. The short intron 4-5 of *VEGF* gene, holding conserved 5′ and 3′ splice sites, was selected and incorporated into the recombinant construct. Thus, this is important to join PCR fragments without introducing any undesired nucleotide in order to proper splicing. In this way, the unique features of *Eco*31I enzyme and specific design of NNNN sequence are very crucial. The recognition sites of *Kpn*I and *Bgl*II restriction enzymes were introduced in F1, R2 primers, respectively, which are used to facilitate the cloning of the VEGF-111 recombinant cDNA. In addition, 18-bp sequence of the exon 8a was also incorporated before *Bgl*II recognition site in R2 primer (Fig. 1).

The sequence of exons 1–4 was amplified through the first PCR reaction. PCR was performed in 25-μl reaction mixture containing standard 1× PCR buffer (20 mM Tris-HCl pH 8.6, 50 mM KCl, CinnaGen Inc, Iran), 120 ng of the cDNA template, 100 pmol of each primers (F1, R1), 0.4 mM mix dNTPs (CinnaGen Inc, Iran), 2.5 U Taq DNA polymerase (CinnaGen Inc, Iran), 1.5 mM $MgCl_2$ (CinnaGen Inc, Iran). The PCR program was as follows: initial denaturation for 5 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 54–66 °C, 72 °C for 20 s, and a final extension step of 10 min at 72 °C for. Second, PCR was carried out to amplify the introns 4–5 and exon 8a in a volume of 25 μl, which included 50 ng of the chromosomal DNA extracted from human blood cells, 0.4 pmol/μl of each primers (F2, R2), 0.4 mM mix dNTPs (CinnaGenInc, Iran), 2.5 U Taq DNA polymerase (CinnaGen Inc, Iran), 2.0 mM $MgCl_2$ (CinnaGen Inc, Iran), and 10× PCR buffer (20 mM Tris-HCl pH 8.6, 50 mM KCl) (CinnaGenInc, Iran). The PCR program was carried out with the following temperature profile: an initial step at 95 °C for 7 min, 30 cycles at 95 °C for 30 s, 66–72 °C for 30 s, and 72 °C for 20 s and a final step at 72 °C for 7 min. PCR products were detected on 2 % agarose gel.
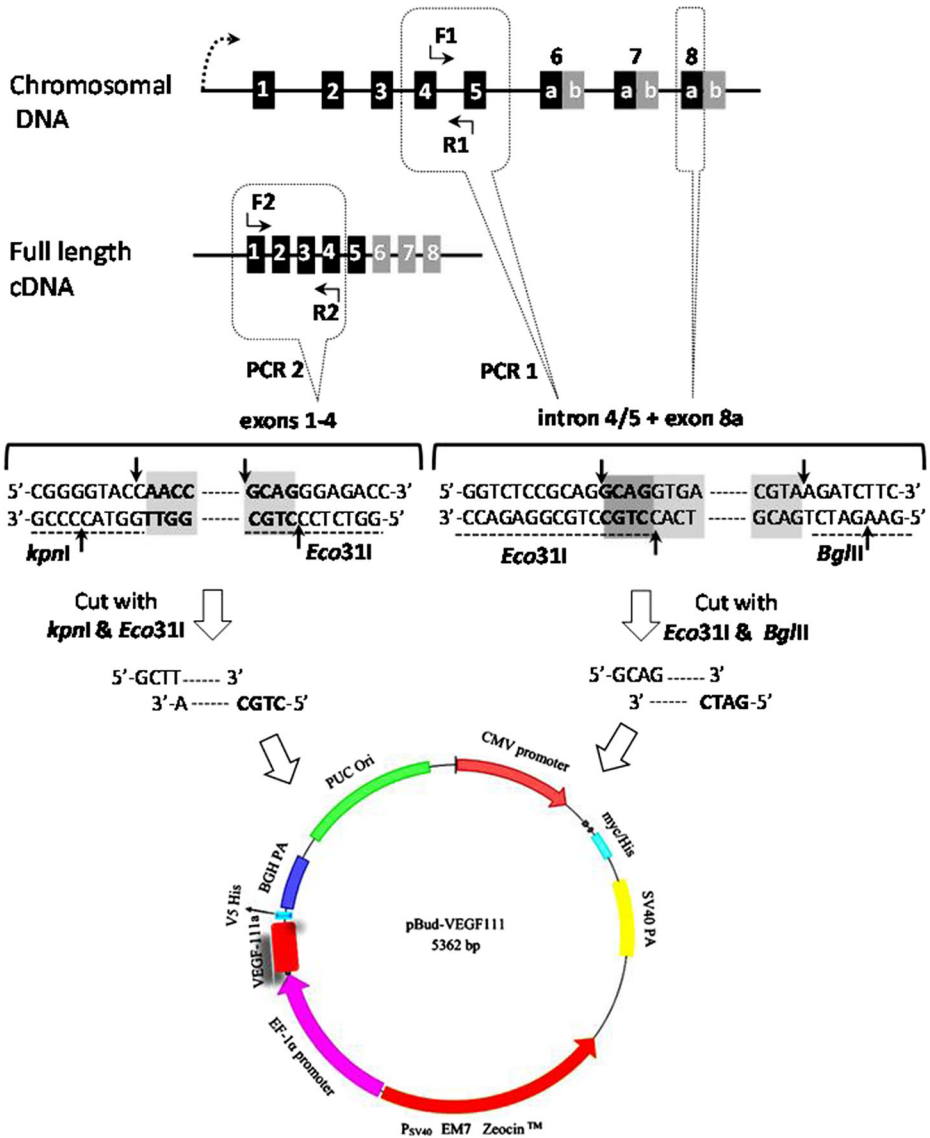
Fig. 1 Schematic illustration of the procedure for the synthesis of the recombinant VEGF-111 cDNA and production of pBud-VEGF-111 plasmid. VEGF-111 contains exons 1–4 and 8a. The first PCR was done to amplify the intronic sequence (intron 4/5) of VEGF-A gene (PCR1). Exons 1–4 were amplified through the second PCR (PCR2). *Curved arrow* indicates the origin of transcription. This map has not been drawn to the scale. *The underlined sequences* indicate the restriction sites of enzymes which were introduced by the primers. The starting and ending bases of two fragments are *shaded gray*

Cloning of VEGF-111 cDNA

The products of the first and second PCR were subjected to double digestion with *Kpn*I, *Eco*31I, and *Bgl*II, *Eco*31I restriction enzymes (Fermrntas, Germany), respectively, at 37 °C for 2 h. The double digested fragments were purified from a 2 % agarose gel by a Fermentas

Table 1 The primers used for generation of recombinant VEGF-111 cDNA

Amplified fragments	Primers	Primer sequence
Exons 1–4	F2	5′-GGggtaccAACCATGAACTTTCTGCTGTCT-3′
	R2	5′-ATTggtctccCTGCATTCACATTTGTTGTGCTGTA-3′
Intron 4–5	F1	5′-AATggtctccGCAGGTGAGGATGTAGTCACGGATT-3′
	R1	5′-AagatctCCGCCTCGGCTTGTCACACTGCAATG GGTAGGGAGAGAG-3′

Note: The restriction sites for *Kpn*I and *Bgl*II are underlined. The restriction sites for *Eco*31I are shaded black. The bold bases "GCAG" in F1 are originated from the last four bases of the sense strand in exon 4 and complementary to the bold bases in R2

gel extraction kit (Germany). The plasmid pBud.CE4.1 (Invitrogen, Carlsbad, CA, USA) was digested by *Kpn*I and *Bgl*II prior to gel purification. The ligation reaction was performed at 16 °C for 16 h in a total volume of 20 μl containing 30 ng of each of the two enzyme-digested segments, 50 ng of the double digested pBud.CE4.1, and 3 U T4 DNA ligase (Fermentas). This ligation mixture was directly used for transformation of the competent cells of *E. coli* Top 10 (Invitrogen, USA). Then, the transformed cells were plated on an LBA plate containing 50-μg/ml Zeocin antibiotic (Invitrogen, Carlsbad, CA, USA). In the next step, two colonies were picked randomly, and plasmids were extracted and cut using *Kpn*I and *Bgl*II to confirm the presence of the desired recombinant VEGF-111 cDNA. Finally, dideoxy termination sequencing with the ABI automated sequencer was used to confirm the authenticity of the constructed gene. All molecular methods were carried out according to the standard methods and procedures [19].

Cell Culture and Transient Transfection

In order to study the expression of VEGF-111, two mammalian cell lines HEK 293 and CHO-*dhfr* − (dihydrofolate reductase-negative) were purchased from the Pasteur Institute, IRAN. CHO-*dhfr* − cells were cultured in Dulbecco's modified Eagle's medium (DMEM, High Glucose, GlutaMAX) with 10 % fetal bovine serum (FBS), 100 mg/ml streptomycin and 0.1 mM hypoxanthine (Sigma), 0.016 mM thymidine (Sigma), and 0.002 mM MTX (methotrexate) (Sigma) at 37 °C, 97 % humidity, and 5 % $CO2$. HEK 293 cells were cultured in DMEM, high glucose with 10 % FBS, and 100 mg/ml streptomycin. Before transfection, HEK 293 and CHO-*dhfr* − cells were transferred in a 12-well plate. When the density of HEK 293 and CHO-*dhfr* − cells reached 80–90 %, the recombinant eukaryotic expression plasmid (pBud-VEGF111) was transfected into the cells using the Lipofectamine kit (Lipofectamine LTX with Plus Reagent) (Invitrogen, USA) according to the manufacturer's instructions. The empty vector pBud.CE4.1 was also transfected into HEK 293 and CHO-*dhfr* − cells as negative control. The cells were incubated with the transfection mixture for 6 h at 37 °C in the 5 % $CO2$. At the end of incubation, fresh medium was supplemented and incubated for an additional 48 h. After further incubation, HEK 293 and CHO-*dhfr* − cells were collected for evaluation of the recombinant VEGF-111 expression.

RNA Extraction and RT-PCR Assay

Total RNA was extracted from the transfected HEK 293 and CHO-*dhfr* − cells by RNeasy mini Kits (Qiagen, Hilden, Germany) following the manufacture-recommended protocol. The RNA quantity, sufficient amount of RNA for cDNA synthesis, and RNA quality were

confirmed by spectrophotometer and electrophoresis. After reverse transcription with hexameric random primers using a RevertAid First Strand cDNA Synthesis Kit (Fermentas), aliquots of products were subjected to real-time PCR analysis. Eukaryotic translation elongation factor 1 alpha 1 (*EEF1A1*) and beta-actin (*ACTB*) were used as reference genes, respectively, in HEK 293 and CHO-*dhfr* − cells. Specific primers (BIONEER, Korea) were designed using AlleleID7.8 and Oligo7 software for VEGF-111, *EEF1A1*, and *ACTB* gene (Table 2). Real-time PCR was performed using Applied Biosystems Real-Time PCR device and Fermentas kit (Maxima SYBR Green qPCR Master Mix (2X), ROX Solution provided). PCR were carried out in a total volume of 25 μl containing 0.4 pmol/μl from each primer, 2× SYBR Mix (12.5 μl), 4 U Taq DNA polymerase, and 30 ng/μl cDNA. 2X SYBR Mix contains PCR buffer, MgCl$_2$, dNTPs, and SYBR Green. The PCR program was as follows: initial denaturation for 5 min at 95 °C followed by 33 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C and a 50 s at 72 °C. Fluorescent measurements were taken after all extension steps. Each sample was run in two replicates, and the mean Ct values were used for further evaluation. The relative gene expression of *VEGF-111* was calculated by the $2^{-\Delta\Delta Ct}$ method [20].

Dot Blot and ELISA Detection

The VEGF-111 was inserted into pBud.CE4.1 plasmid under the control of human elongation factor 1α-subunit (EF-1α) promoter for high-level, constitutive, and independent expression. PBud.CE4.1 contains a C-terminal peptides encoding a polyhistidine (6xHis) metal-binding tag for detection and purification of recombinant proteins. The presence of His6-tagged recombinant VEGF-111 in transfected HEK 293 and CHO-*dhfr* − cells was monitored by an anti-His antibody (monoclonal anti-polyhistidine peroxidase conjugate, Sigma-Aldrich) using dot blot and ELISA techniques. Cell lysate was prepared by homogenization in RLT lysis which contains 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % SDS, 1 % sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride (PMSF).

Dot blot was carried out by spotting 3 μl of each cell lysates from either transfected HEK 293 or CHO-*dhfr*−, 1/1000 dilution of anti-His antibody (positive control), and untransfected HEK 293, CHO-*dhfr* − cell lysate (negative control) onto nitrocellulose membranes and drying at the room temperature. The nitrocellulose membrane was blocked in 5 ml of Block buffer for 2 h at the room temperature, followed by washing twice for 5 min with 50 ml of PBST (PBS, 0.1 % Tween-20). The membrane was hybridized with 5 ml of 1:1000 diluted anti-His antibody for 2 h, followed by three washes for 5 min. Finally, the membrane was developed using Amersham ECL Prime detection reagent for 1 min and exposed to radiography film in a dark room. ELISA was performed as the manufacturer instructions. Briefly, the plates were coated with 50 μl of cell lysate in 50-μl coating buffer and incubated at 4 °C overnight. Two percent PBS and untransfected HEK 293, CHO-*dhfr* − cell lysate and purified His-tagged

Table 2 Real-time PCR primers

Primer	Primer sequence	Tm	PCR product length
VEGF F	5′ GCTACTGCCATCCAATCGAG 3′	59.4	**188 bp**
VEGF R	5′ GGTTTGATCCGCATAATCTGCAT 3′	58.9	
EEF1A1 F	5′ CCCTTCTGGCTTACACACT 3′	56.7	**225 bp**
EEF1A1 R	5′ TGAACCAAGGCATGTTAGCAC 3′	57.9	
ACTB F	5′ CCAACCGTGAAAAGATGACC 3′	57.3	**124 bp**
ACTB R	5′ GAGTCCATCACAATGCCAGT 3′	57.3	

recombinant human growth hormone (hGH, 1 mg/ml, in 1/10,000 dilution) were used as negative and positive control, respectively. The coated plates were washed three times with wash buffer. Then, each well of the plate was blocked with 5 ml of PBS 1X+ 10 % skim milk for 2 h. Next, the plates were incubated with 100 µl of 1:1000 diluted anti-His antibody for 2 h. The color was then developed by adding 100 µl tetramethyl benzidine (TMB, Cyto Matin Gene, Isfahan, Iran) to each well in the dark and at the room temperature for 15 min. The reaction was stopped with 100-µl stop solution (Cyto Matin Gene, Isfahan, Iran). The absorbance at 450 nm was read in an ELISA reader with a 630-nm diffraction filter.

Results

Purity and Integrity of Extracted Total RNA

Purified RNA from breast tumor tissue had an A260/A280 ratio (optical density ratio at 260 and 280 nm) of 2 in 10 mM Tris-HCl, pH 7.5. Two sharp and clear bands of 28S and 18S rRNA were detected on a 1 % agarose gel, confirming the integrity of the extracted RNA for reverse transcription (data not shown). The cDNA of purified RNA were synthesized and used for real-time PCR analysis. According to the validation assays, 0.4 pmol/µl primers and 30 ng/µl cDNA were optimal for amplification exons 1–4 fragment.

Recombinant VEGF-111 cDNA Construction

In this work, the *Eco*31I restriction enzyme was employed to synthesize recombinant VEGF-111 cDNA. Two fragments of VEGF-111 cDNA were successfully amplified using gradient PCR that was also used to determine the optimal annealing temperature. The 417-bp fragment of exons 1–4 was amplified in the first gradient PCR. The best temperature for obtaining products of outstanding quality was 66 °C. The second gradient PCR for amplification of intron 4/5 and exon 8a (393 bp) was carried out at an optimal annealing temperature of 66 °C. The final products of the first and second PCRs were double digested using *Kpn*I-*Eco*31I and *Eco*31I-*Bgl*II, respectively, to produce complementary cohesive ends (3′-CGTC-5′ and 5′-GCAG-3′). Then, three fragments, the double-digested linearized pBud.CE4.1 vector, exons 1–4, and intron 4/5 + exon 8a were purified. Finally, multicomponent ligation was performed, and the resulting DNA was transformed into Top10 competent cells. The transformed colonies were confirmed with double digestion of the isolated recombinant pBud-VEGF111 plasmid using *Kpn*I and *Bgl*II. The presence of an 809-bp band on a 1 % agarose gel after double digestion indicated that the two separately amplified fragments were successfully and precisely ligated into the pBud.CE4.1 plasmid (Fig. 2). Sequencing with the ABI automated sequencer confirmed the authenticity of the constructed gene.

Transfection and RNA Extraction of HEK 293 and CHO-*dhfr* − Cells

Recombinant pBud-VEGF111 plasmid was transiently transfected into two mammalian expression systems, HEK 293 and CHO-*dhfr* − cells. Isolation of total RNA was performed using trypsinized cells 48 h after transfection. Then, the RNA quantity and quality were confirmed using a spectrophotometer and gel electrophoresis (Fig. 3a). All extracted RNAs from each cell line had an A260/A280 ratio of 1.9–2.1, which was suitable for cDNA synthesis.
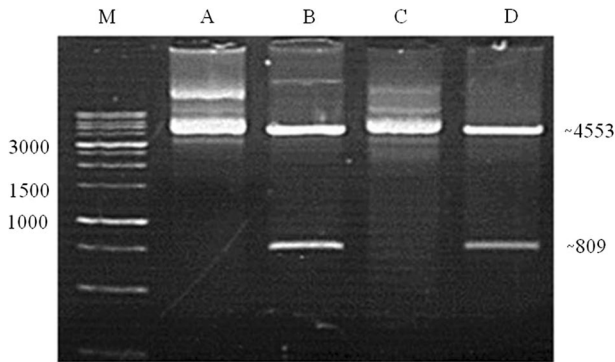
Fig. 2 Confirmation of the structure of the recombinant pBudVEGF-111 plasmid with restriction digestion analysis. pBudVEGF-111 plasmid was isolated from the transfected cells and digested with *Kpn*I and *Bgl*II. The expected 809-bp fragment was seen following cleavage of the construct. DNA marker (*lane M*), undigested pBud-VEGF-111 construct (*lane A and C*), PBud-VEGF-111 digested with *Kpn*I /*Bgl*II (*lane B and D*). A 2 % TAE agarose gel was used here for gel electrophoresis by staining with 2 mg/ml of ethidium bromide (EB). Samples (3 μl) were mixed with 1 μl lof 6x DNA loading buffer per lane. All of the numbers are in bp

VEGF-111 Expression in HEK 293 and CHO-*dhfr* − Cells

HEK 293 and CHO-*dhfr* − cells were transfected with recombinant pBud-VEGF111 for 48 h, and the level of mRNA was evaluated with RT-PCR. To validate the RT-PCR conditions, the synthesized cDNA was used to assess the specificity of the designed primers. According to the validation assays, 0.5 pmol/μl primers and 20 ng/μl cDNA were optimal for amplification of *VEGF-111* and the constitutively expressed housekeeping genes *EEF1A1* and *ACTB*. Gradient
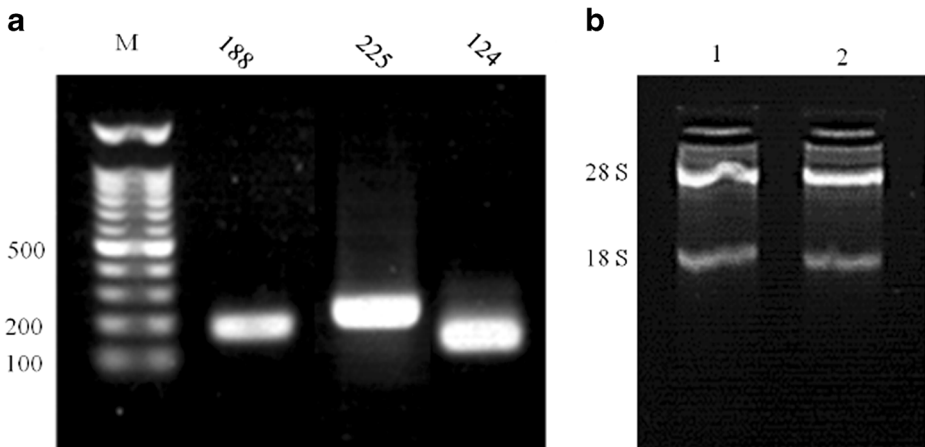

Fig. 3 The amplified products of PCR reactions and integrity of extracted RNA. **a** *Lane M* (DNA marker), *lane 1* (specific PCR product of *VEGF* gene), *lane 2* (specific PCR product of *EEF1A1*), and *lane 3* (specific PCR product of *ACTB*) in 55 °C. There is no primer dimer formation. All of the fragment sizes are in base pair (bp). A 2 % agarose gel was used here for gel electrophoresis. **b** After RNA isolation from transfected HEK 293 (*lane 1*) and CHO-dhfr⁻ (*lane 2*), the integrity of purified RNA was examined by agarose gel electrophoresis and ethidium bromide staining. 28S and 18S rRNA bands were observed as well as expected intensity bands ratio which was approximately 2:1, respectively

PCR was performed at a range of 54–60 °C for the three primer sets. The best results were obtained at 55 °C with no detection of nonspecific PCR products or primer dimmers (Fig. 3b).

Quantitative RT-PCR and Expression of *VEGF-111*

For real-time PCR analysis, *EEF1A1* and *ACTB* were amplified from cDNA from HEK 293 and CHO-*dhfr* − cells, respectively, to normalize the amount of total RNA in each sample. Transfected HEK 293 and CHO-*dhfr* − cell lines were evaluated with real-time PCR to investigate the amount of recombinant *VEGF-111* expression in the transfected cells. Two independent experiments were performed to determine the relative expression values of *VEGF-111* compared with the reference gene in each cell line. We also determined the expression of endogenous *VEGF* in untransfected HEK 293 and CHO-*dhfr* − cells compared with the expression of transfected *VEGF-111* in HEK 293 and CHO-*dhfr* − cells. The results, which are shown in Table 3 and Fig. 4, demonstrate a high level of *VEGF-111* expression both in HEK 293 and CHO-*dhfr* − cells. Interestingly, *VEGF-111* expression was increased 18- and 10-fold in transfected HEK 293 and CHO-*dhfr* − cells, respectively, compared with untransfected cells. Recombinant *VEGF-111* levels were compared with endogenous *VEGF* to generate the fold increase in expression, because the primer sets used here for VEGF-111 amplification were designed on exons 1–4 which are common in all VEGF isoforms. Electrophoresis of RT-PCR products from transfected cells showed the expected fragments of recombinant *VEGF-111* (188 bp), *EEF1A1* (225 bp), and *ACTB* (124 bp) (Fig. 5), indicating that transcriptional expression of the construct was successful.

Detection of VEGF-111 with Dot Blotting and ELISA

Recombinant pBud-VEGF111-His6 plasmid, containing the entire VEGF-111 protein coding region and suitable start and stop codons, was expressed in two mammalian expression systems to enable appropriate glycosylation of VEGF-111. The presence of His_6-tagged VEGF-111 in transfected HEK 293 and CHO-*dhfr* − cells was monitored using a His tag antibody and dot blotting and ELISA. Dot blot samples containing transfected HEK 293 and CHO-*dhfr* − cells were detected with the antibody, whereas the untransfected HEK 293 and CHO-*dhfr* − cells were used as negative controls and were not detected with anti-His (Fig. 6). His-tagged recombinant human growth hormone was used as the positive control and was detected with anti-His, as expected. ELISA was also used to confirm the production of VEGF-111 by the two different mammalian cell lines to determine the cell type that produced the

Table 3 $2^{-\Delta\Delta Ct}$ analyses for calculating *VEGF* gene expression

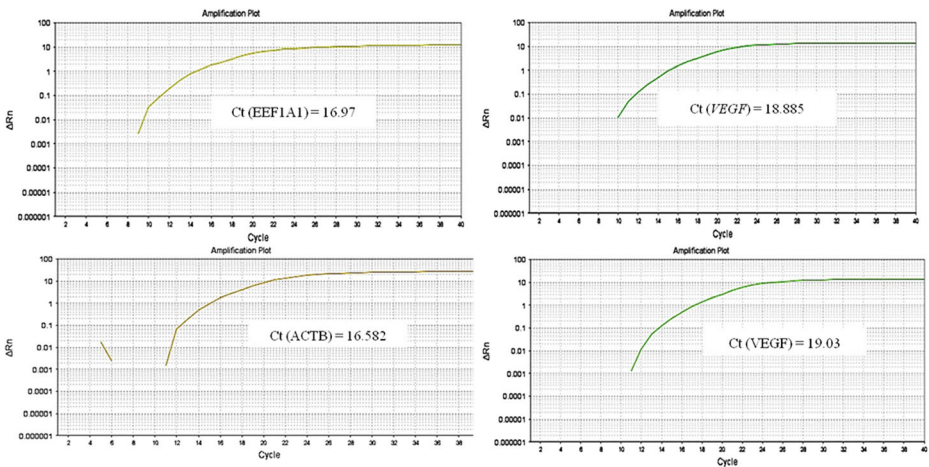| Sample	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
CHO VEGF-111a (ACTB)	14.043		
CHO VEGF-111a (VEGF)	17.03	2.987	-3.3	9.84915
HEK VEGF-111a (EEF1A1)	15.97		
HEK VEGF-111a (VEGF)	19.8	3.21	-4.22	18.6357
CHO control (ACTB)	15.16		
CHO control (VEGF)	21.447	6.287	0	1
HEK control (EEF1A1)	15.81		
HEK control (VEGF)	23.24	7.43	0	1

Fig. 4 The amplification curve and Ct value of *VEGF*, *EEF1A1*, and *ACTB* genes in transfected HEK 293 (**a**) and CHO-*dhfr⁻* (**b**) samples

higher level. ELISA confirmed the production of VEGF-111 in transfected HEK 293 and CHO-*dhfr* − cells compared to untransfected cells and phosphate-buffered saline (PBS). The triplet values were averaged, and ODs were calculated. The absorbance at 450 nm were 0.432 ± 0.02, 0.463 ± 0.027, 0.443 ± 0.024, 3.045 ± 0.073, 1.37 ± 0.17, and 1.84 ± 0.075 in PBS, untransfected HEK 293 cells, untransfected CHO-*dhfr* − cells, the positive control, transfected CHO-*dhfr* − cells, and transfected HEK 293 cells, respectively. ELISA was only performed to detect VEGF-111, and quantitative analysis will be performed in future studies.

Discussion

Recombinant DNA technology and production of recombinant proteins that are key regulatory factors in different biological processes are required for research in medical biotechnology [21]. Angiogenesis is a key biological process in many physiological and pathological
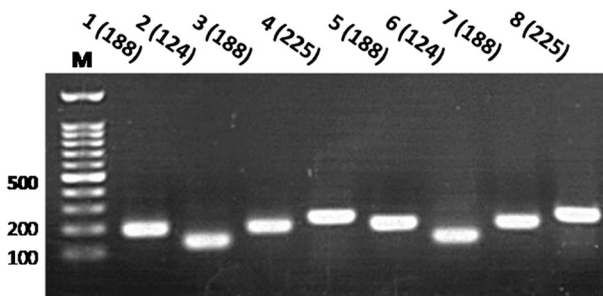

Fig. 5 Amplification of *VEGF*, *EEF1A1*, and *ACTB* genes by RT PCR. *Lane M* (DNA marker), *lane 1*, *2* and *lane 5*, *6* (specific PCR product of *VEGF* (188 bp) and *ACTB* (124 bp) genes) in transfected and untransfected CHO-*dhfr-* cells, respectively. Two specific bands for *VEGF* (188 bp) and EEF1A1 genes (225 bp) were detected in transfected (*lane 3*, *4*) and untransfected (*lane 7*, *8*) HEK 293 cells. There is no primer dimer formation. All of the fragment sizes are in base pair (bp). A 2 % TAE agarose gel was used here for gel electrophoresis by staining with 2 mg/ml of ethidium bromide (EB)
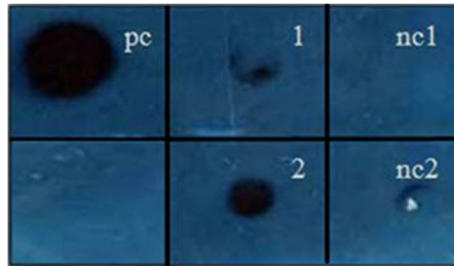
Fig. 6 In vitro expression analysis of the construct in HEK 293 and CHO-*dhfr*- cells by dot blotting. Dot blot analysis of HEK 293 and CHO-*dhfr* − cells. Evaluation of the transfected CHO-*dhfr* − (1) and HEK 293 (2) cell lines revealed the expression of VEGF-111 (which contains exons 4, 5, and 8). (pc) positive control, His-tagged recombinant human growth hormone; untransfected HEK 293 (nc2), CHO-*dhfr* − (nc1) cell lysate as negative control. Immunoblotting analysis was performed using anti-His6-tag monoclonal antibody

conditions, and several factors are involved in regulation of this process. Among these factors, VEGF is essential for the initiation and regulation of angiogenesis [22]. Currently, VEGFs are widely produced using efficient recombinant DNA technology methods by well-known companies such as Millipore, R&D Systems, and Life Technologies. Recombinant VEGFs have been used in many studies of angiogenesis, stem cells, regenerative medicine, and tissue engineering [23, 24]. VEGF-165 was the first described isoform of VEGF-A. VEGF-165 was widely investigated and is a predominant regulator of angiogenesis in health and disease. In 2002, Zhou and colleagues created expression constructs of two isoforms, VEGF-165 and VEGF-121, to examine their properties. Three years later, a research group cloned the VEGF-165 isoform into a eukaryotic expression vector and reported new information about the influences of VEGF-165 on the proliferation ability of vascular endothelial cells [25, 26]. Subsequently, other VEGF-A isoforms have been discovered and cloned. Among all VEGF varieties, the newly identified VEGF-111 with strong angiogenic activity is highly diffusible and resistant to proteolysis. VEGF-111 is not expressed in any healthy human or murine cells, but its expression is induced by UV-B and genotoxic drugs, which may participate in rendering cancer cells resistant to anticancer drugs [9]. On the other hand, the unique characteristics of VEGF-111 make it a potential target for new treatments for conditions in which angiogenesis is deficient. Also, elevation of VEGF mRNA expression occurs in these pathological situations, but the proteolytic environment and enzymes, such as plasmin and matrix metalloproteinases, degrade VEGF and prevent angiogenesis. From a therapeutic point of view, the use of different VEGF isoforms for the treatment of heart attacks, burns, and chronic wounds such as venous, pressure, and diabetic ulcers has been investigated. VEGF-111 may overcome the above limitations and serve as a promising therapeutic agent.

Recent studies have evaluated the effects of VEGF-111 in the tendon healing process and angiogenesis following ovarian tissue xenotransplantation. Injection of VEGF-111 stimulates the tendon healing process in patients with tendon lesions, which are the most frequent type of sports injury. VEGF-111 improves blood vessel recruitment and the viability of the ovarian cortex by decreasing ischemia [9, 27].

Owing to the unique properties of VEGF-111 and its beneficial use in research and the clinic, we designed and synthesized VEGF-111 cDNA. In addition, we improved the level of VEGF-111 expression in mammalian cell lines by inserting an intronic sequence within the coding region; quantitative analysis will be performed in future studies [28, 29]. Recombinant VEGF was successfully synthesized using the high-efficiency class IIs restriction enzyme-based method, which is simple, minimally error prone, and inexpensive [30, 16, 31]. The

conserved 5′- and 3′-splice site sequences of the intron are very important for appropriate splicing. In this regard, the joining of PCR fragments comprising exons 1–4 and intron 4/5 plus exon 8a results in a naturally occurring desired sequence without the addition of any undesired nucleotides. In the present study, the pBud-VEGF111 recombinant plasmid was transfected into both HEK 293 and CHO-*dhfr* − eukaryotic cell lines to compare the expression level of VEGF-111. Inappropriate folding and glycosylation are two features that could change the properties of our recombinant growth factors. To reduce the possibility of these undesired effects, VEGF-111 was produced in eukaryotic cells. HEK 293 cells produce more suitable posttranslational modifications compared with CHO-*dhfr* − cells, which produce nonhuman glycosylation. CHO-*dhfr* − cells produce different glycans at the N-terminal position of the recombinant growth factor, increasing the risk of immunogenicity in humans. We were able to produce VEGF-111 in both cell types, and this VEGF-111 may be useful in research and the clinic. CHO cells are most commonly used for long-term (stable) gene expression and when high yields of heterologous proteins are required. About 140 recombinant proteins are currently approved for therapeutic use, most produced in CHO cells that have been adapted for growth in high-density suspension cultures, and many more are in clinical trials. Although, CHO-dhfr − cells produce different glycans at the N-terminal position of the recombinant growth factor, we are able to produce VEGF-111 in CHO cells which may be useful in research. In fact, because the promoter used to express VEGF-111 was the human *EF-1α* promoter, VEGF-111 expression may be higher in human HEK 293 cells owing to the greater compatibility of the *EF-1α* promoter in this cell line. The results of real-time PCR showed that the *EF-1α* promoter cloned into the pBud.CE4.1 vector was highly active and that VEGF expression in both transfected cell lines was significantly increased. VEGF expression was increased about 10- and 18-fold in transfected CHO-*dhfr* − and HEK 293 cells, respectively, compared with untransfected cells. *VEGF* expression in human cell lines was almost two times more than expression in CHO-*dhfr* − cells, suggesting that the promoter is used more efficiently in human cells. Dot blotting and ELISA were used to detect VEGF-111 expression, confirming that the recombinant protein was produced in both cell lines. In conclusion, we successfully expressed recombinant VEGF-111 in two eukaryotic cell lines and will improve and optimize its production in future studies.

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