Expression and characterization of functional human vascular endothelial growth factor ($VEGF_{165}$) in *Kluyveromyces lactis*

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Abstract

Among the vascular endothelial growth factor (VEGF) family variants, the 165-amino acid isoform ($VEGF_{165}$) is the best characterized and most potent endothelial cell mitogenic factor. It is known that $VEGF_{165}$ mediates angiogenesis and has the potential for the therapeutic applications. In this study, the expression system of *Kluyveromyces lactis* that produces the recombinant human $VEGF_{165}$ has been evaluated. The gene encoding human $VEGF_{165}$ was successfully cloned in the pKLAC2 expression vector containing a strong LAC4 promoter, after which a pKLAC2-$VEGF_{165}$ plasmid was constructed. After the transformation, the recombinant human vascular endothelial growth factor 165 ($rhVEGF_{165}$) was expressed in *K. lactis* GG799 cells (~ 5.7 mg/L) confirmed by SDS-PAGE and Western blotting and downstream purification processed comprising ammonium sulphate precipitation and affinity chromatography. The biological activity of the purified $rhVEGF_{165}$ was confirmed by the proliferation of the human umbilical vein-derived endothelial cells (HUVEC) in a dose- and time-dependent manner. The *K. lactis*-derived $rhVEGF_{165}$ exhibited a higher proliferative activity compared with a commercially available $rhVEGF_{165}$ with a half-maximal effective concentration of 3.0. Cell migration analysis was conducted to evaluate the *in vitro* wound healing effect of the produced $rhVEGF_{165}$ *via* a scratch assay. These findings indicate that *K. lactis* could be a suitable host for secreting bioactive human $VEGF_{165}$ for therapeutic use.

Introduction

Vascular endothelial growth factor (VEGF) is a family of protein that requires specific mitogen for the growth of endothelial cells (Leung et al. 1989). It plays a significant role in fundamental processes, such as growth of new capillaries from the existing vasculature vessels (angiogenesis) and formation of blood vessels from endothelial precursor cells (vasculogenesis) (Ferrara and Kerbel 2005). Originally, VEGF was identified as a vascular permeability factor (VPF) (Senger et al. 1983; Dvorak et al. 2006) and then Ferrara and Henzel (1989) named as VEGF.

The VEGF family belongs to the platelet-derived growth factor (PDGF) and comprises of the following: VEGF-A, placental growth factor, VEGF-B, VEGF-C, VEGF-D, VEGF-E and VEGF-F (Li and Eriksson 2001; Shibuya 2003). VEGF-A, which is generally referred to as VEGF, is the founding member of the family; it characterizes eight exons. Due to the alternative splicing of exons 6 and 7 of VEGF pre-mRNA, various isoforms are generated, such as $VEGF_{121}$, $VEGF_{121b}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{165b}$, $VEGF_{189}$ and $VEGF_{206}$ (Tischer et al. 1991; Ferrara 2004; Holmes and Zachary 2005). All these isoforms have a common amino terminal domain consisting of 110 amino acids, while the length of carboxyl-terminal differs.

$VEGF_{165}$ is the first VEGF splice variant of the VEGF-A family that has been described. It has a molecular weight of 38.2 kDa and a theoretical pI of 8.6 (Keck et al. 1997). The disulphide-linked homodimeric protein $VEGF_{165}$ is the most abundant and best-characterized isoform that is comprised of two 165 amino acid polypeptide chain monomers (Ferrara 1991). Each of the two monomers has a single glycosylation site and a cystine-knot motif. $VEGF_{165}$ digestion by plasmin yields two domains: amino

terminal homodimer (amino acids 1–110), which contains the VEGF receptor binding site for the kinase domain receptor (KDR), and the fms-like tyrosine kinase receptor (Flt-1). The carboxyl-terminal portion of the $VEGF_{165}$ (amino acids 111–165) is known as the carboxyl-terminal heparin-binding domain; it mediates heparin affinity, which is important for the mitogenic effect on the endothelial cells (Keyt et al. 1996; Takahashi and Shibuya 2005). Numerous different cells and tissues are known to express $VEGF_{165}$ as a strong mitogenic factor under physiological and pathological conditions (Lauer et al. 2002).

VEGF mainly contributes to vascular development through angiogenesis by inducing endothelial cell proliferation and migration. Moreover, it regulates these functions by binding to specific tyrosine kinase VEGF receptors (VEGFRs) on the surface of vascular endothelial cells. The activation of VEGF-A with VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk1 in mice) is necessary for angiogenesis and vascular permeability under both physiological and pathological conditions. Conversely, at the early stage of embryogenesis and in the development of lymphatic vessels, VEGF-C/D and its receptor VEGFR-3 mainly regulate angiogenesis (Shibuya 1995; Shibuya 2006; Alitalo and Carmeliet 2002).

The function of VEGF is not limited to angiogenesis; VEGF also plays a significant role in normal physiological functions, such as wound healing, menstrual cycle, bone formation, haematopoiesis and neural development (Gerber et al. 1999; Ferrara et al. 1996; Chintalgattu et al. 2003; Reichardt and Tomaselli 1991). Moreover, it can be released in high amounts by activated platelets and increase the formation of granulation tissue at the wound site (Hoeben et al. 2004; Dvorak et al. 1995). Under stress conditions, such as hypoxia, chemotherapy and radiotherapy, VEGF acts as a survival factor secreted by tumour cells (Riedel et al. 2004; Gorski et al. 1999, Scott et al. 1998). The formation of new vessels causes tumour cells to enter the circulation and increase metastasis. Due to its role in tumour formation and survival strategies, VEGF is considered to be an attractive target for anti-angiogenic medication (Gardner et al. 2007). VEGF and its receptors are required for embryonic angiogenesis and the development of vascular network (Ferrara et al. 1996, Tammela et al. 2005). In normal adult health, VEGF plays a significant role in non-malignant diseases, such as rheumatoid arthritis (Lee et al. 2001), psoriasis (Xia et al. 2003), diabetes and ischemic retinopathies (Aiello et al. 1995).

VEGF is synthesized by numerous cell types, including endothelial cells, tumour cells, fibroblasts, platelets, macrophages, neutrophils, keratinocytes, T cells and renal mesangial cells (Boocock et al. 1995; Sunderkotter et al. 1994, Verheul et al. 1997; Frank et al. 1995; Iijima et al. 1993). Aside from bacterial expression (Pizarro et al. 2010; Nguyen et al. 2016), several eukaryotic expression host systems have been employed for the recombinant production of VEGF, including yeast (Mohanraj et al. 1995; Kang et al. 2013), Chinese hamster ovary cells (CHOs) (Lee et al. 2008), insect cells (Lee et al. 2006), transgenic rice (Chung et al. 2014) and silkworm (Wu et al. 2004). The most preferred system is *Escherichia coli (E. coli)*; however, it exhibits drawbacks, such as the tendency to form inclusion bodies, protein misfolding and difficult purification steps (Nguyen et al. 2016). In addition, yeast and bacteria are cost-effective systems, unlike other higher organisms, such as insects or mammalian cells; however, unlike bacteria, yeasts do not require refolding for activity.

Kluyveromyces lactis (K. lactis) is an emerging yeast host for the production of heterologous protein. It has the ability to obtain high levels of protein, thus making it particularly suitable for industrial applications. In addition to being used as a host for protein expression, *K. lactis* has potential for numerous biotechnological applications, for example, as an infant formula component, protein supplement, flavour enhancer, commercial enzyme producer, lactase and lactic acid source and probiotic single-cell protein (Bonekamp and Oosterom 1994; Belem and Lee 1998; Oishi et al. 1999; Colussi et al. 2005; Porro et al. 1999; Ghaly et al. 2005).

Moreover, *K. lactis* has all the advantages of other yeast expression systems, such as the following: it has the ability to obtain high culture densities and high yield; it exhibits posttranslational modification mechanisms similar to mammals; it has the ability to form disulphide linkages; and it can be used for scalable fermentation and for the production of endotoxins and carcinogens (Kim and Kim 2016). The genome of *K. lactis* is completely sequenced; thus, it can be easily manipulated (Dujon et al. 2004). It is also possible to use both integrative and episomal expression vectors for the *K. lactis* expression. The status generally recognized as safe (GRAS), a designation provided by the US Food and Drug Administration, makes *K. lactis* particularly safe and useful in food and feed applications. For enzymes and proteins expressed from *K. lactis*, endotoxin analysis is not required (Bonekamp and Oosterom 1994). A standard inexpensive culture medium is sufficient for the rapid growth of *K. lactis*, and high-molecular-weight proteins can be secreted in the culture medium. For industrial applications, the induction of recombinant protein in *K. lactis* is important as it has constitutive promoters and also does not require expensive or highly flammable chemicals, such as methanol as in *P. pastoris.* Therefore, the use of explosion-proof equipment is not necessary (Rocha et al. 2011; Rosa et al. 2013).

In *K. lactis*, approximately 100 heterologous proteins have been successfully produced and many of these proteins are commercially available in different industries (Spohner et al. 2016). The achievement of the use of *K. lactis* as a host for protein expression in the food industry has continued in the large-scale production of therapeutic proteins in the pharmaceutical industry. Numerous pharmaceutical proteins, such as interleukin 1-β, insulin precursor, interferon-α, β-lactoglobulin, macrophage colony-stimulating factor (M-CSF) and various antibodies, have been produced in *K. lactis* (Fleer et al. 1991; Feng et al. 1997; Chen et al. 1992; Rocha et al. 1996; Hua et al. 1994; Swennen et al. 2002).

In this study, the gene of the human $VEGF_{165}$ was inserted into the *K. lactis* pKLAC2 expression vector and transformed into the *K. lactis* GG799 yeast cells. The secreted $rhVEGF_{165}$ was purified from the culture medium *via* affinity chromatography. In addition, analysis of the bioactivity of the produced $VEGF_{165}$ was conducted by testing *in vitro* for proliferation and wound healing assay.

Materials And Methods

Strains, plasmids and growth conditions

pET22b plasmid containing the human $VEGF_{165}$ cDNA (GenBank accession no. AF486837.1) was obtained from Biomatik (Biomatik Corporation, Cambridge, Canada). *E. coli* DH5a was utilized for the maintenance and manipulation of plasmids. The *K. lactis* Protein Expression Kit (New England BioLabs, Massachusetts, ABD) containing the pKLAC2 expression vector was utilized. Moreover, the *E. coli* DH5a cells were grown in the Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl and pH 7.0) at a temperature of 37 °C. The *K. lactis* GG799 cells were grown in the YPGlu medium (1% yeast extract, 2% peptone and 2% glucose) at 30 °C in a shaker at 200 rpm. In addition, expression of the transformed *K. lactis* GG799 cells was performed in the YPGal medium (1% yeast extract, 2% peptone and 4% galactose). All the chemicals were purchased from Sigma–Aldrich (Darmstadt, Germany) and Merck (Merck Millipore, Darmstadt, Germany).

Table 1. List of strains, plasmids, and oligonucleotide primers in this study

	Relevant characteristics
Strains	
E. coli DH5α	F^- *recA1 endA1 hsdR17(*rk^- mk^+*) supE44 thi-1 gyrA relA1*
K. lactis GG799	*MAT*α [pGKl1$^+$]
Plasmids	
pET22b-$VEGF_{165}$	pET22b carrying full-length $VEGF_{165}$ gene, Ap^R
pKLAC2	Expression vector, Ap^R
pKLAC2-$VEGF_{165}$	pKLAC2 carrying full-length $VEGF_{165}$ gene, Ap^R
Primers	
*Xho*I- $VEGF_{165}$ (forward)	5′-TTTTCTCGAG*AAAAGA*ATGAATTTCTTACTTTC-3′
*EcoR*I- $VEGF_{165}$ (reverse)	5′-TTTTGAATTCTCA**GTGGTGGTGGTGGTGGTGGTG**-3′

Restriction sites have been underlined. Ap^R means ampicillin resistance. Reverse primer ends with His-tag (shown in bold). Forward primer contains Kex protease cleavage site (italics).

Isolation of the human $VEGF_{165}$ sequence

The *Homo sapien*s $VEGF_{165}$ mRNA was optimized for the expression in *K. lactis* yeast cells with the use of online databases, the Codon Adaptation Tool (http://www.jcat. de/). Using the Codon Optimization Tool (https://eu.idtdna.com/CodonOpt), the codon usage of $VEGF_{165}$ was analysed. Based on the known codon bias of *K. lactis*, low-usage codons were replaced by high-usage codons (http://www.kazusa.jp/codon/cgi-bin/showcodon.cgi?species=28985). The optimized sequence inserted

into the pET22b plasmid was commercially obtained. The sequence encoding $VEGF_{165}$ was amplified by polymerase chain reaction (PCR) using the pET22b-$VEGF_{165}$ as a template. The forward primer included an *Xho*I restriction site and a sequence encoding the Kex protease processing site. To generate an in-frame fusion between the a-mating factor (a-MF) secretion leader sequence and the $VEGF_{165}$ sequence, the Kex protease site was added in a primer sequence. The reverse primer included an *EcoR*I restriction site, a stop codon (TCA) and the sequence encoding a hexahistidine C-terminal tag. PCR was performed in 50 ml master mix containing 10X *Pfu* polymerase buffer, 1.5 mM $MgCl_2$, 0.3 mM dNTPs, 1unit of *Pfu* polymerase (Takara, Tokyo, Japan) and 200 nmol of forward and reverse primers. The thermal cycling parameters used for PCR were as follows: 95 °C for 60 s, 65 °C for 60 s, 72 °C for 60 s and extension at 72 °C for 5 min. After 35 cycles, the amplified $VEGF_{165}$ was isolated from a 2% (w/v) agarose gel and then purified with the GENEAID® gel extraction system (Geneaid, Taiwan) according to the manufacturer's instruction. A 1 kb DNA ladder (HyperLadder, Bioline Reagents Limited, UK) was used to estimate the sizes of the PCR product of the $VEGF_{165}$.

Construction of the recombinant expression vector

The purified PCR product of $VEGF_{165}$ and *K. lactis* pKLAC2 expression plasmid DNA were separately digested with *Xho*I and *EcoR*I (Takara, Tokyo, Japan) restriction enzymes. The $VEGF_{165}$ insert was ligated into the pKLAC2 vector using T4 DNA ligase (Promega, USA). Moreover, the strain *E. coli* DH5a was transformed using the recombinant expression vector, pKLAC2- $VEGF_{165}$, by heat shock (2 min at 42 °C). Selection of the positive transformants was performed by evaluating their ampicillin antibiotic resistance owing to the presence of ampicillin-resistant gene in the *K. lactis* pKLAC2. The plasmid DNA was isolated (Geneaid ® Presto™ Mini Plasmid Kit, Taiwan) and then analysed by PCR. Then, restriction digestion was performed to confirm $VEGF_{165}$ cloning.

The generation of recombinant *K. lactis* yeast cells

The strain *K. lactis* GG799 was utilized as the host for $VEGF_{165}$ expression. *K. lactis* GG799 cells were grown in the YPGlu medium at 30 °C in a shaker at 200 rpm until the optical density at 600 nm reached 0.6. Then, the cells were pelleted at 1,000 × g for 5 min at 4 °C, washed twice with cold sterile H_2O and centrifuged again. The cells were washed again and resuspended in cold and sterile electroporation buffer (pH 8.0, YPD including 10 mM DTT, 40 mM HEPES) and kept on ice until the transformation. To generate an expression cassette, 10 mg of plasmid DNA $VEGF_{165}$-pKLAC2 was linearized by digesting with *Sac*II (Takara, Tokyo, Japan). The competent *K. lactis* yeast cells were incubated in the presence of the linearized plasmid DNA, and transformation was achieved *via* electroporation using pulse discharges (2.1 ms, 2.5 kV, Eppendorf Eporator, Multiporator, Hamburg, Germany). The cells were incubated in outgrowth medium (YPD including 1 M sorbitol) for 1 h at 30 °C (without shaking) for recovering after electroporation and spread on yeast carbon base (YCB) (BD Difco, Sparks, MD, USA) agar medium plates (3% 1 M Tris-HCl pH 7.4, 1.17% YCB and 2% agar) containing 5 mM acetamide for 3 days at 30 °C. Then transformants on plates were transferred to the YCB medium and incubated for 2 days at 30 °C at 200

rpm. The genomic DNA of yeast cells was isolated according to the method described by Harju et al. (2004). The positive transformant containing the $VEGF_{165}$ sequence was screened by PCR using specific primers (used before for cloning) and then analysed by agarose gel electrophoresis. In addition, the positive transformants were grown in the YPGal medium (1% yeast extract, 2% peptone, 4% galactose), and the protein expression levels of the culture medium samples were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to determine the best clone that secreted the $VEGF_{165}$. The recombinant *K. lactis* clone in the YPGal medium was stored by adding 60% (v/v) glycerol and freezing at −80 °C.

Expression of recombinant human $VEGF_{165}$ in *K. lactis* GG799

The starter culture of the $VEGF_{165}$ producer *K. lactis* GG799 clone was grown for 2 days at 30 °C in 5 ml YCB medium containing 5 mM acetamide. The growth culture was inoculated at a ratio of 1:100 into 300 ml YPGal medium (4% galactose) and was allowed to grow at 30 °C for 3 days. Cell harvesting was performed by centrifugation at 6,000 × g for 45 min after incubation.

Purification ofhuman $VEGF_{165}$

The cell-free supernatant of the transformed *K. lactis* containing secreted recombinant human $VEGF_{165}$ were precipitated with 60% ammonium sulphate solution in an ice bath for 60 min and then centrifuged at 10,000 × g for 20 min. Then, the protein pellet was resuspended in 100 mM phosphate buffer (pH 7.5) and was subjected to affinity chromatography for purification through the binding of the hexahistidine tag (located at the C-terminal end of the $VEGF_{165}$) using a 0.5 ml nickel–nitrilotriacetic acid (Ni–NTA) resin column (Qiagen, Hilden, Germany). To remove non-specifically bound impurities, the column was washed with phosphate buffer. The $VEGF_{165}$ was eluted with phosphate buffer containing 300 mM imidazole. The eluted protein was then transferred to the dialysis tubing containing 1000 kDa MWCO (Spectrum Laboratories, California, USA) and dialysed against 20 mM HEPES (pH 7.4) at 4 °C. The protein concentration was quantified *via* absorbance spectroscopy at 280 nm using an extinction coefficient of 24,950 $M^{-1}cm^{-1}$.

Western blot analysis

The protein samples were subjected to electrophoresis under the denaturing conditions using 12% SDS gel stained with Coomassie Brilliant Blue R-250, as described by Laemmli (1970). The protein bands on the gel were transferred onto the polyvinylidene difluoride (PVDF) membrane for Western blot analysis. The membrane was blocked with 5% (w/v) skim milk in phosphate-buffered saline (PBS) for 30 min at room temperature. The membrane blot was washed and rinsed with PBS and incubated with 6xHis-Tag (C-term)/AP Monoclonal Antibody (Invitrogen, Carlsbad, CA, USA) at 1:3000 dilution for 2 h at room temperature. After washing with blocking buffer for three times, the membrane was incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solutions (BCIP/NBT) (Sigma–Aldrich, Darmstadt, Germany) in colour development buffer.

Proliferation assay and scratch assay

Human umbilical vein endothelial cells (HUVECs) (gift from Bingöl University, Department of Molecular Biology and Genetic) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Biochrom, Berlin, Germany) supplemented with 15% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine and 0.1% gentamicin sulphate at 37 °C in a 5% CO_2 humidified atmosphere. Assay for cell proliferation was performed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate the biological activity of the produced $VEGF_{165}$. Then, HUVECs were seeded in 96-well plates at a density of 4×10^3 per well and treated with the purified $VEGF_{165}$ and the commercial *E. coli*-derived $VEGF_{165}$ (Abcam, Cambridge, UK) at various concentrations for 2 and 6 days. The untreated cells were incubated as a negative control to determine the relative number of the treated cells. Three replicates for each concentration were performed. After cell culture, the cells were incubated with MTT (5 mg/mL) for 4 h. The culture media were removed, and the obtained formazan crystals were solubilized in dimethyl sulfoxide. The absorbance of each well was read at 570 nm using a microplate reader. Experiments were repeated in triplicate and expressed as mean ± standard error (SE). The group means were compared using Duncan's test or a one-way analysis of variance (ANOVA). A p value < 0.05 was considered as statistically significant.

In vitro scratch wound assay

Scratch assay was performed to determine the effect of $VEGF_{165}$ on wound healing. L929 mouse fibroblast cells were seeded in a 6-well plate (10^4 cells/well) in the DMEM culture medium and grown to confluence. Then confluent cell layers were scratched with a pipette tip, and cellular debris was removed by washing off with PBS. The cells were treated with DMEM with 15% FBS containing 5 and 10 ng/mL of *E. coli*-derived and *K. lactis*-derived $VEGF_{165}$. The controls received only fresh DMEM. The cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere. Then, an image of the scratch area was captured using a phase-contrast inverted microscope (4X magnification, Olympus CKX41), and the percentage of the scratch area was analysed using an Olympus analysis software.

Results And Discussion

Construction of the pKLAC2-$VEGF_{165}$ expression vector

To achieve protein secretion, the $VEGF_{165}$ gene was cloned downstream of the *K. lactis* α-MF secretion domain, which resulted in the expression of an α-MF fusion protein. The human $VEGF_{165}$ gene was amplified from the pET22b-$VEGF_{165}$ vector using gene-specific primers. Gradient PCR was performed to optimize the annealing temperature (65 °C) (Additional file 1: Figure S1). The amplified $VEGF_{165}$ and pKLAC2 plasmid DNA were separately digested with restriction enzymes (*Xho*I and *EcoR*I) and ligated with each other to generate pKLAC2-$VEGF_{165}$ (Fig. 1A). *E. coli* DH5a cells were transformed with the resulting recombinant plasmid and incubated on LB agar medium, including ampicillin (100 mg/mL). A

total of 12 single colonies were randomly selected. The plasmid DNA of the clones analysed by PCR and three of these clones revealed the PCR product of the 633 bp $VEGF_{165}$. Based on the results of the restriction enzyme digestion with *Nco*I–*EcoR*I and *Hind*III–*Eco*RI enzyme couples, only one clone (clone 2) was used for the transformation into *K. lactis* (Additional file 1: Figure S2).

Expression of $VEGF_{165}$ in *K. lactis*

The *Sac*II-linearized plasmid pKLAC2-$VEGF_{165}$ was transformed into competent *K. lactis* GG799 cells *via* electroporation. The expression cassette was targeted for insertion into the LAC4 promoter region of the *K. lactis* chromosome by the 3' and 5' ends of the $P_{LAC4\text{-}PBI}$ promoter by homologous recombination. pKLAC2 contains a variant of the strong *K. lactis* lactase LAC4 promoter, which is located in the vector pKLAC2 and acetamidase gene (amdS) for the positive selection of the transformed strains in a nitrogen-free medium. This medium contains acetamide as the only nitrogen source. A total of 14 transformants were randomly selected, and integration of the expression cassette into the *K. lactis* genome was confirmed by genomic DNA PCR, with the use of specific primers of the $VEGF_{165}$ gene (Fig. 1C). The $VEGF_{165}$ gene (633 bp) was observed in 12 of these transformants (except for clone 2 and clone 4 in Fig. 1C). $VEGF_{165}$ secretion was evaluated using small-scale expression assays in 5 mL YCB medium containing 5 mM acetamide. Then, SDS-PAGE analysis of the culture supernatant was conducted to confirm the presence of $VEGF_{165}$. Four positive transformants that successfully secreted the target protein were analysed by nucleotide sequencing to confirm the integration of the recombinant expression cassette into the *K. lactis* GG799 chromosome (Additional file 1: Figure S3).

The positive clone that exhibits the highest homology found in the GenBank database was grown in 300 ml YPGal medium. The $VEGF_{165}$ was expressed-fused with α-MF secretion domain and processed in the Golgi complex. Subsequently, it was secreted in the fermentation medium. The fusion proteins were cleaved by Kex protease at the processing site (KR↓) between the α-MF domain and N-terminus of the $VEGF_{165}$ gene. The supernatant of the medium was precipitated, and then, the precipitant solved in phosphate buffer was loaded to Nickel affinity chromatography. The eluted recombinant human $VEGF_{165}$ was subjected to 12% (w/v) SDS-PAGE under reducing conditions and visualized by Coomassie blue staining. The non-glycosylated recombinant human $VEGF_{165}$ monomer has a MW of approximately 20 kDa as reported in previous studies (Fiebich et al. 1993; Catena et al. 2010; Peretz et al. 1992). As presented in Fig. 2, the MW of $VEGF_{165}$ was approximately 30 kDa as a result of adding tags (restriction enzyme sites and His-tag). Moreover, $VEGF_{165}$ glycosylation varies according to the host organism and the effects of the MW of the protein. It was reported that the CHO-derived $VEGF_{165}$ exhibits higher MW compared with the *E. coli*-derived and insect cell-derived $VEGF_{165}$ due to different glycosylation mechanisms (Lee et al. 2008). The yield of the produced recombinant human $VEGF_{165}$ was determined by measuring the absorbance at 280 nm and using an extinction coefficient of 24950 $M^{-1}cm^{-1}$. The highest expression level of the analysed clones was 5.7 mg/L of $VEGF_{165}$. It has been reported that recombinant human $VEGF_{165}$ is expressed in various expression systems, such as *E. coli* at 1.5 mg/L

(Taktak-BenAmar et al. 2017) and *Saccharomyces cerevisiae* at 4 mg/L (Kang et al. 2013). In other studies, the expression levels of 80 mg/L (Lee et al. 2008) for CHO cell cultures and 20 mg/L (Lee et al. 2006) for insect cells at were achieved. The yields in previous studies related recombinant heterologous protein production such as human IFN-γ (2 mg/L) and hepatitis C virus E2 glycoprotein (1 mg/L) are comparable to the results of this study (Spohner et al. 2016).

Biological activity of the produced $VEGF_{165}$

To confirm the biological activity of the $hVEGF_{165}$ protein expressed and purified in this study, cell proliferation assay was performed by MTT assay. The growth of the HUVECs was stimulated by $rhVEGF_{165}$ in a dose-dependent manner. As presented in Fig. 3A, recombinant human $VEGF_{165}$ significantly enhanced the proliferation of HUVEC cells for 2 days ($p < 0.0001$). The results revealed that $VEGF_{165}$ produced in *K. lactis* exhibits higher mitogenic activity than the commercial *E. coli*-derived human $VEGF_{165}$ for all $VEGF_{165}$ concentrations (Additional file 1: Table S1). Particularly, 2.5 ng/mL of $VEGF_{165}$ exhibited the greatest proliferative activity (2.83% cell viability) ($p < 0.001$), which was higher than that of the commercial $VEGF_{165}$ (2.64%) (Additional file 1: Table S2). Accordingly, this concentration was employed in the subsequent time-dependent assay. The proliferative effect for 2 and 6 days was compared with absorption at 570 nm. After 6 days, the rate of proliferation was increased, and the produced $VEGF_{165}$ exhibited higher proliferation in the absorbance of 0.91 compared with the commercial $VEGF_{165}$ (0.85). In general, the half-maximal effective concentration (EC_{50}) of commercial $rhVEGF_{165}$ has been reported as 1–6 ng/mL. In this study, the EC_{50} of the *K. lactis*-derived $rhVEGF_{165}$ protein was calculated to be approximately 3.0 ng/mL, which means a similar bioactivity to previous studies in CHO cells and insect cells (Lee et al. 2008; Lee et al. 2006).

L929 mouse fibroblast cells treated with $VEGF_{165}$ were utilized in the wound healing assay. The $VEGF_{165}$ was found to facilitate wound healing at 5 and 10 ng/mL concentrations (Fig. 4) compared with the untreated control. Moreover, it has a similar effect to the commercial *E. coli*-derived $VEGF_{165}$ (Additional file 1: Table S3). It was clearly observed that, after 24 and 48 h of incubation at a concentration of 10 ng/mL, the percentages of the wound site treated with *K. lactis*-derived $VEGF_{165}$ were 69,54% and 85,71%, respectively. Meanwhile, the percentages of the cell-covered area were 73,48% and 93,12% for cells treated with *E. coli*-derived $VEGF_{165}$ and 55,24% and 78,59% for untreated cells. The migration assay confirmed that $VEGF_{165}$ is biologically active and that the ability of L929 mouse fibroblast cells to heal wound was promoted by $VEGF_{165.}$ These results indicated that the proliferation efficiency of *K. lactis*-derived $VEGF_{165}$ on HUVECs was higher than that of the commercial *E. coli*-derived $VEGF_{165}$. Contrarily, the produced $VEGF_{165}$ influenced the migration of L929 mouse fibroblast cells at a slower rate compared with *E. coli*-derived $VEGF_{165.}$

Conclusion

In this study, the expression of biologically active rhVEGF$_{165}$ protein in the *K. lactis* expression system, which has been used extensively as a host for heterologous protein expression, was successfully established. The recombinant human VEGF$_{165}$ was expressed in *K. lactis* GG799 cells and secreted in the culture medium. The VEGF$_{165}$ was purified functionally active from *K. lactis* culture medium by ammonium sulphate precipitation, Ni-affinity chromatography and dialysis. The produced *K. lactis*-derived VEGF$_{165}$ exhibited higher biological activity than the commercially available *E. coli*-derived VEGF$_{165}$. Moreover, it stimulated the proliferation of HUVECs. However, the migration effect of the rhVEGF$_{165}$ was tested on L929 mouse fibroblast cells. The addition of rhVEGF$_{165}$ to cell culture media led to a significant increase in migration, which contributed to an accelerated rate of wound healing in an *in vitro* scratch wound model when compared with untreated controls. In total, these observations indicate that rhVEGF$_{165}$ can be exploited as a therapeutic agent for the basic investigation of physiological states, such as wound healing and several diseases related to VEGF$_{165}$.

Abbreviations

VEGF, vascular endothelial growth factor; VEGF$_{165}$, vascular endothelial growth factor 165; rhVEGF$_{165}$, recombinant human vascular endothelial growth factor 165; *K. lactis*, *Kluyveromyces lactis*; HUVEC, human umbilical vein derived endothelial cells; PDGF, platelet-derived growth factor; KDR, kinase domain receptor; Flt-1, fms-like tyrosine kinase receptor; VEGFRs, VEGF receptors; CHOs, Chinese hamster ovary cells; *E. coli, Escherichia coli*; GRAS, generally recognized as safe; M-CSF, macrophage colony-stimulating factor; LB, Luria–Bertani; PCR, polymerase chain reaction; a-MF, a-mating factor; YCB, yeast carbon base; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; Ni–NTA, nickel–nitrilotriacetic acid; PVDF, polyvinylidene difluoride; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solutions; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ANOVA, one-way analysis of variance; EC$_{50}$, half-maximal effective concentration.

Declarations

Ethics approval and consent to participate

All the authors have read and agreed to the ethics for publishing the manuscript.

Consent for publication

The authors approved the consent for publishing the manuscript.

Availability of data and materials

The authors approved the availability of data and materials for publishing the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HKC, IG and SET designed the experiment and analyzed the data. HKC and SET performed all of experiments. HKC wrote the manuscript. All authors read and approved the final manuscript.

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Figures

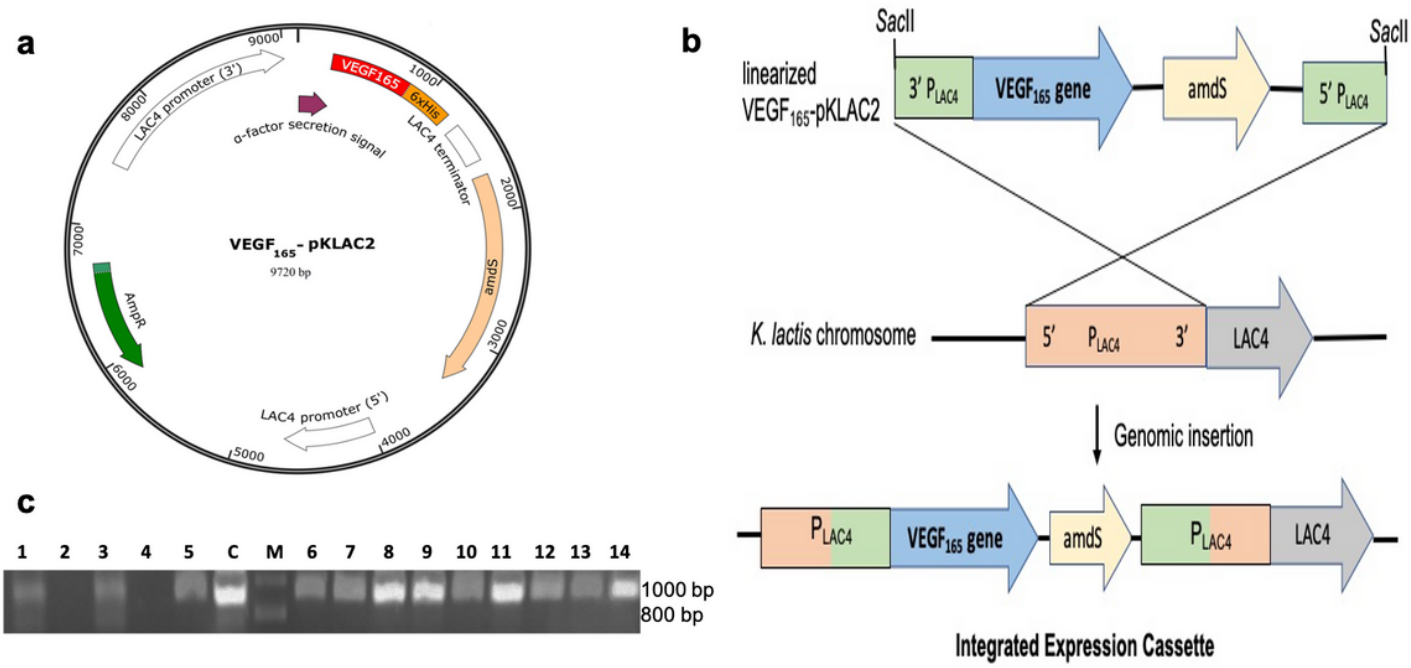

Figure 1

Construction of the pKLAC2-VEGF165 expression vector and integration into the K. lactis genome. a) Schematic diagram of the VEGF165 recombinant expression vector based on the pKLAC2. b) Genomic integration of linear pKLAC2-VEGF165 expression cassette into the K. lactis genome. c) Confirmation of the integration of the expression cassettes into the K. lactis genome by PCR. The numbers correspond to the randomly selected K. lactis clones and “c” to the amplification product using pET22b-VEGF165 plasmid as a positive control.

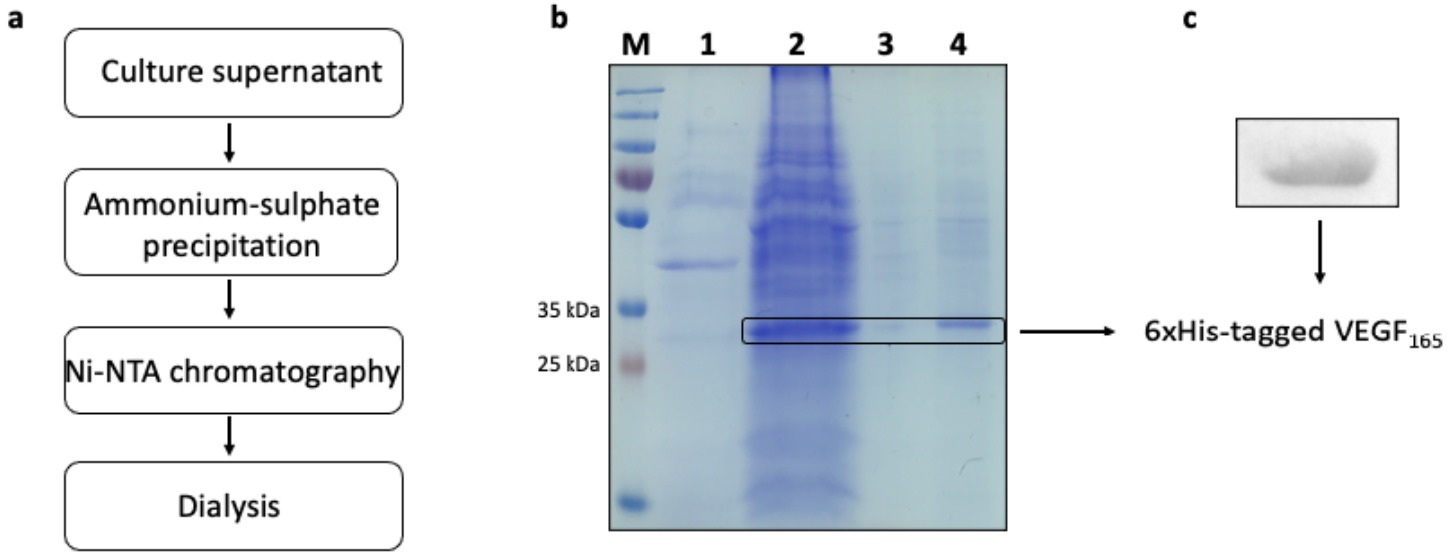

Figure 2

Purification of recombinant 6XHis-tagged VEGF165 from K. lactis. a) Schematic diagram of the purification process. b) SDS-PAGE (12% Tris-glycine gel) analysis of purified fraction recombinant human VEGF165. M, molecular-weight marker in kDa. The spent culture medium of untransformed K. lactis strain GG799 (negative control) was analysed after ammonium sulphate precipitation (Lane 1) and affinity chromatography (Lane 3). The recombinant human VEGF165 in the spent culture medium of transformed K. lactis strain GG799 with pKLAC2-VEGF165 expression cassette following ammonium sulphate precipitation (Lane 2) and eluted VEGF165 following affinity chromatography (Lane 4). c) Western blot analysis of purified VEGF165 with an anti-His antibody.

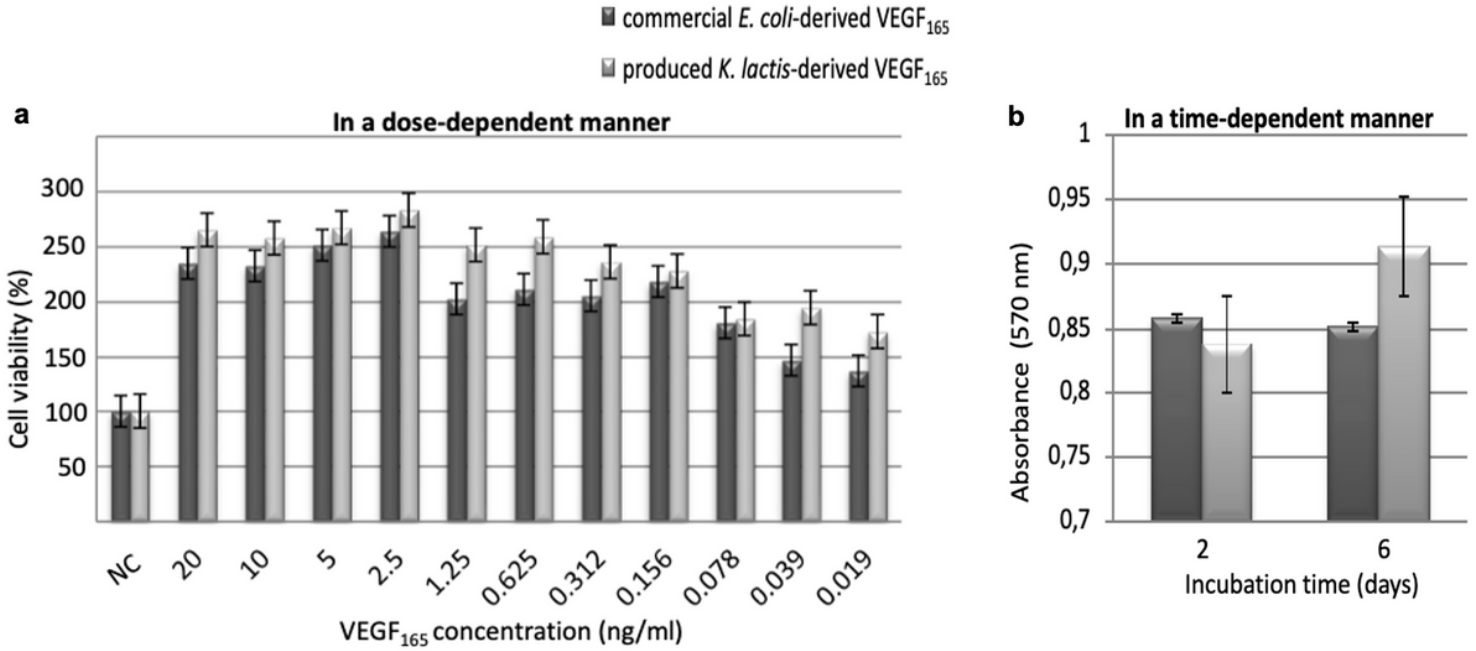

Figure 3

The dose- and time-dependent effects of the produced VEGF165 and commercially available VEGF165 on the proliferative activity of HUVECs. MTT assay was performed. a) In a dose-dependent test, HUVECs were seeded and incubated at 37 °C for 5 days with various VEGF165 concentrations. b) In a time-dependent test, HUVECs were seeded and incubated at 37 °C for 2 or 6 days with 2.5 ng/mL of VEGF165. The results are presented as cell viability (%) and absorbance (570 nm).

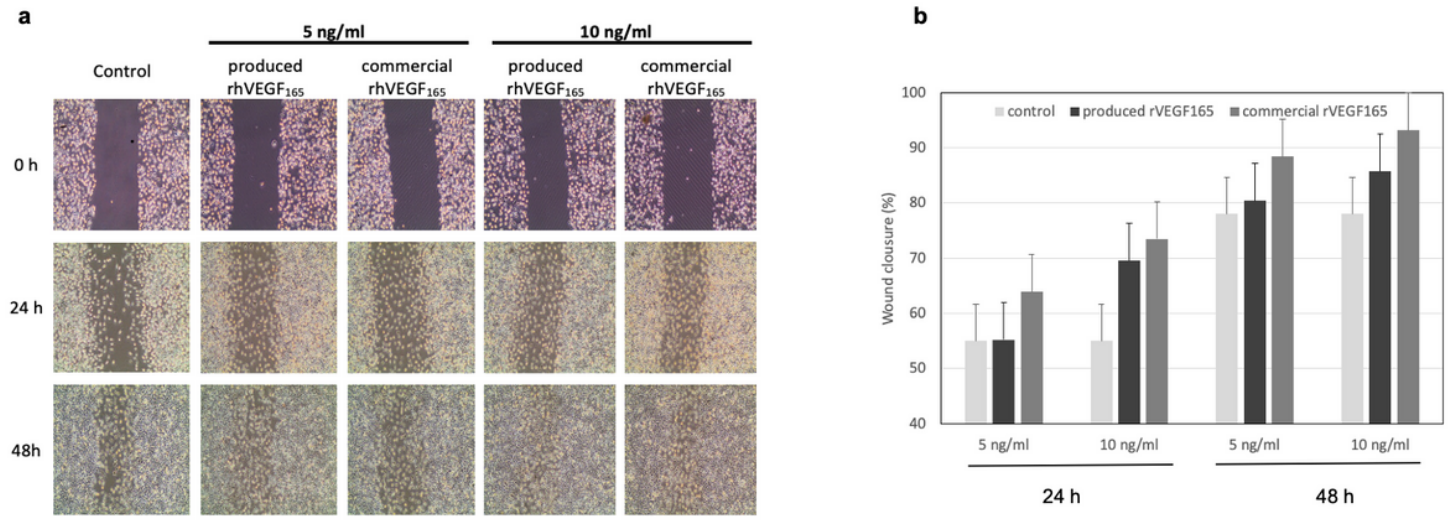

Figure 4

Effect of K. lactis-derived VEGF165 on scratch-wound assays. Cell migration was determined in wound healing scratch experiments. a) Representative images from the wound healing assay of L929 mouse fibroblast cell cultures treated with 5 and 10 ng/mL of rhVEGF165. Data are expressed as mean ± S.D. (n = 3) at 24 and 48 h. Statistical significance compared with the control (untreated cells) and commercial E. coli-derived VEGF165. b) Summary graph showing the percentage of the cell-covered area.

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