Expression and characterization of functional human vascular endothelial growth factor (VEGF165) in Kluyveromyces lactis

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Abstract

Among the vascular endothelial growth factor (VEGF) family variants, the 165-amino acid isoform (VEGF\textsubscript{165}) is the best characterized and most potent endothelial cell mitogenic factor. It is known that VEGF\textsubscript{165} mediates angiogenesis and has the potential for the therapeutic applications. In this study, the expression system of \textit{Kluyveromyces lactis} that produces the recombinant human VEGF\textsubscript{165} has been evaluated. The gene encoding human VEGF\textsubscript{165} was successfully cloned in the pKLAC2 expression vector containing a strong LAC4 promoter, after which a pKLAC2-VEGF\textsubscript{165} plasmid was constructed. After the transformation, the recombinant human vascular endothelial growth factor 165 (rhVEGF\textsubscript{165}) was expressed in \textit{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\textsubscript{165} was confirmed by the proliferation of the human umbilical vein-derived endothelial cells (HUVEC) in a dose- and time-dependent manner. The \textit{K. lactis}-derived rhVEGF\textsubscript{165} exhibited a higher proliferative activity compared with a commercially available rhVEGF\textsubscript{165} with a half-maximal effective concentration of 3.0. Cell migration analysis was conducted to evaluate the \textit{in vitro} wound healing effect of the produced rhVEGF\textsubscript{165} via a scratch assay. These findings indicate that \textit{K. lactis} could be a suitable host for secreting bioactive human VEGF\textsubscript{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\textsubscript{121}, VEGF\textsubscript{121b}, VEGF\textsubscript{145}, VEGF\textsubscript{165}, VEGF\textsubscript{165b}, VEGF\textsubscript{189} and VEGF\textsubscript{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\textsubscript{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 pl of 8.6 (Keck et al. 1997). The disulphide-linked homodimeric protein VEGF\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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 \textit{Escherichia coli} (\textit{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 (Spohnert 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<sub>165</sub> was inserted into the K. lactis pKLAC2 expression vector and transformed into the K. lactis GG799 yeast cells. The secreted rhVEGF<sub>165</sub> was purified from the culture medium via affinity chromatography. In addition, analysis of the bioactivity of the produced VEGF<sub>165</sub> 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<sub>165</sub> cDNA (GenBank accession no. AF486837.1) was obtained from Biomatik (Biomatik Corporation, Cambridge, Canada). <i>E. coli</i> DH5α was utilized for the maintenance and manipulation of plasmids. The <i>K. lactis</i> Protein Expression Kit (New England BioLabs, Massachusetts, ABD) containing the pKLAC2 expression vector was utilized. Moreover, the <i>E. coli</i> DH5α 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 <i>K. lactis</i> 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 <i>K. lactis</i> 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**               |
| <i>E. coli</i> DH5α       | F<sup>−</sup> recA1 endA1 hsdR17(rk<sup>−</sup> mk<sup>+</sup>) supE44 thi-1 gyrA relA1 |
| <i>K. lactis</i> GG799    | MATα [pGKI1<sup>+</sup>]                                  |
| **Plasmids**              |
| pET22b-VEGF<sub>165</sub> | pET22b carrying full-length VEGF<sub>165</sub> gene, Ap<sup>R</sup> |
| pKLAC2                   | Expression vector, Ap<sup>R</sup>                        |
| pKLAC2-VEGF<sub>165</sub>| pKLAC2 carrying full-length VEGF<sub>165</sub> gene, Ap<sup>R</sup> |
| **Primers**               |
| Xhol- VEGF<sub>165</sub> (forward) | 5’-TTTTCTCGAGAAAAAGAATGAATTTCTTTACTTTCCC-3’ |
| EcoR<sup>I</sup>- VEGF<sub>165</sub> (reverse) | 5’-TTTTGAATTCAGTGGTGTTGGTGTTGGTGTTGGTGTTGGTGTTGGTG-3’ |

Restriction sites have been underlined. Ap<sup>R</sup> 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<sub>165</sub> sequence**

The <i>Homo sapiens</i> VEGF<sub>165</sub> mRNA was optimized for the expression in <i>K. lactis</i> 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<sub>165</sub> was analysed. Based on the known codon bias of <i>K. lactis</i>, 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\textsubscript{165} was amplified by polymerase chain reaction (PCR) using the pET22b-VEGF\textsubscript{165} as a template. The forward primer included an \textit{XhoI} 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\textsubscript{165} sequence, the Kex protease site was added in a primer sequence. The reverse primer included an \textit{EcoRI} 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 \textit{Pfu} polymerase buffer, 1.5 mM MgCl\textsubscript{2}, 0.3 mM dNTPs, 1 unit of \textit{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\textsubscript{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\textsubscript{165}.

**Construction of the recombinant expression vector**

The purified PCR product of VEGF\textsubscript{165} and \textit{K. lactis} pKLAC2 expression plasmid DNA were separately digested with \textit{XhoI} and \textit{EcoRI} (Takara, Tokyo, Japan) restriction enzymes. The VEGF\textsubscript{165} insert was ligated into the pKLAC2 vector using T4 DNA ligase (Promega, USA). Moreover, the strain \textit{E. coli} DH5a was transformed using the recombinant expression vector, pKLAC2- VEGF\textsubscript{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 \textit{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\textsubscript{165} cloning.

**The generation of recombinant \textit{K. lactis} yeast cells**

The strain \textit{K. lactis} GG799 was utilized as the host for VEGF\textsubscript{165} expression. \textit{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 x g for 5 min at 4 °C, washed twice with cold sterile H\textsubscript{2}O 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\textsubscript{165}-pKLAC2 was linearized by digesting with \textit{SacI} (Takara, Tokyo, Japan). The competent \textit{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<sub>165</sub> 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<sub>165</sub>. 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<sub>165</sub> in K. lactis GG799**

The starter culture of the VEGF<sub>165</sub> 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 of human VEGF<sub>165</sub>**

The cell-free supernatant of the transformed K. lactis containing secreted recombinant human VEGF<sub>165</sub> 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<sub>165</sub>) 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<sub>165</sub> 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<sup>−1</sup> cm<sup>−1</sup>.

**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% CO₂ 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\textsubscript{165}. Then, HUVECs were seeded in 96-well plates at a density of $4 \times 10^5$ per well and treated with the purified VEGF\textsubscript{165} and the commercial \textit{E. coli}-derived VEGF\textsubscript{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\textsubscript{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 \textit{E. coli}-derived and \textit{K. lactis}-derived VEGF\textsubscript{165}. The controls received only fresh DMEM. The cells were incubated at 37 °C in a humidified 5% CO₂ 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\textsubscript{165} expression vector

To achieve protein secretion, the VEGF\textsubscript{165} gene was cloned downstream of the \textit{K. lactis} α-MF secretion domain, which resulted in the expression of an α-MF fusion protein. The human VEGF\textsubscript{165} gene was amplified from the pET22b-VEGF\textsubscript{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\textsubscript{165} and pKLAC2 plasmid DNA were separately digested with restriction enzymes (\textit{XhoI} and \textit{EcoRI}) and ligated with each other to generate pKLAC2-VEGF\textsubscript{165} (Fig. 1A). \textit{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 $Ncol$–$EcoRI$ and $HindIII$–$EcoRI$ 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 SacII-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$_{LAC4-PBI}$ 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 $\alpha$-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 $\alpha$-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<sub>165</sub>**

To confirm the biological activity of the hVEGF<sub>165</sub> protein expressed and purified in this study, cell proliferation assay was performed by MTT assay. The growth of the HUVECs was stimulated by rhVEGF<sub>165</sub> in a dose-dependent manner. As presented in Fig. 3A, recombinant human VEGF<sub>165</sub> significantly enhanced the proliferation of HUVEC cells for 2 days ($p < 0.0001$). The results revealed that VEGF<sub>165</sub> produced in K. lactis exhibits higher mitogenic activity than the commercial E. coli-derived human VEGF<sub>165</sub> for all VEGF<sub>165</sub> concentrations (Additional file 1: Table S1). Particularly, 2.5 ng/mL of VEGF<sub>165</sub> exhibited the greatest proliferative activity (2.83% cell viability) ($p < 0.001$), which was higher than that of the commercial VEGF<sub>165</sub> (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<sub>165</sub> exhibited higher proliferation in the absorbance of 0.91 compared with the commercial VEGF<sub>165</sub> (0.85). In general, the half-maximal effective concentration (EC<sub>50</sub>) of commercial rhVEGF<sub>165</sub> has been reported as 1–6 ng/mL. In this study, the EC<sub>50</sub> of the K. lactis-derived rhVEGF<sub>165</sub> 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<sub>165</sub> were utilized in the wound healing assay. The VEGF<sub>165</sub> 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<sub>165</sub> (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<sub>165</sub> 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<sub>165</sub> and 55,24% and 78,59% for untreated cells. The migration assay confirmed that VEGF<sub>165</sub> is biologically active and that the ability of L929 mouse fibroblast cells to heal wound was promoted by VEGF<sub>165</sub>. These results indicated that the proliferation efficiency of K. lactis-derived VEGF<sub>165</sub> on HUVECs was higher than that of the commercial E. coli-derived VEGF<sub>165</sub>. Contrarily, the produced VEGF<sub>165</sub> influenced the migration of L929 mouse fibroblast cells at a slower rate compared with E. coli-derived VEGF<sub>165</sub>.

**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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**References**

Aiello LP, Pierce EA, Foley ED, Takagi H, Chen H, Riddle L, Ferrara N, King GL, Smith LE (1995) Suppression of retinal neovascularization *in vivo* by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. Proc Natl Acad Sci USA 92:10457–10461

Alitalo K, Carmeliet P (2002) Molecular mechanisms of lymphangiogenesis in health and disease. Cancer Cell 1:219–227

Belem MA, Lee BH (1998) Production of bioingredients from *Kluyveromyces marxianus* grown on whey; an alternative. Crit Rev Food Sci Nutr 38:565–598

Bonekamp FJ, Oosterom J (1994) On the safety of *Kluyveromyces lactis*: a review. Appl Microbiol Biotechnol 41:1–3

Boocock CA, Charnock-Jones DS, Sharkey AM, McLaren J, Barker PJ, Wright KA, Twentyman PR, Smith SK (1995) Expression of vascular endothelial growth factor and its receptors flt and KDR in ovarian carcinoma. J Natl Cancer Inst 87(7):506–516

Catena R, Larzabal L, Larrayoz M, Molina E, Hermida J, Agorreta J, Montes R, Pio R, Montuenga L, Calvo A (2010) VEGF121b and VEGF165b are weakly angiogenic isoforms of VEGF-A. Mol Cancer 9:320

Chen X, Gao B, Shi W, Li Y (1992) Expression and secretion of human interferon alpha A in yeast *Kluyveromyces lactis*. Yi Chuan Xue Bao 19:284–288
Chintalgattu V, Nair DM, Katwa LC (2003) Cardiac myofibroblasts: A novel source of vascular endothelial growth factor (VEGF) and its receptors Flt-1 and KDR. J Mol Cell Cardiol 35:277–286

Chung ND, Kim NS, Giap do V, Jang SH, Oh SM, Jang SH, Kim TG, Jang YS, Yang MS (2014) Production of functional human vascular endothelial growth factor (165) in transgenic rice cell suspension cultures. Enzyme Microb Technol 63:58–63

Colussi PA, Specht CA, Taron CH (2005) Characterization of a nucleus-encoded chitinase from the yeast Kluyveromyces lactis. Appl Environ Microbiol 71:2862–2869

Dujon B, Sherman D, Fisher G (2004) Genome evolution in yeasts. Nature 430:35–44

Dvorak HF, Detmar M, Claffey KP, Nagy JA, van de Water L, Senger DR (1995) Vascular permeability factor/vascular endothelial growth factor: an important mediator of angiogenesis in malignancy and inflammation. Int Arch Allergy Immunol 107:233–235.

Dvorak HF (2006) Discovery of vascular permeability factor (VPF). Exp Cell Res 312:522–526

Ferrara N, Henzel WJ (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 161(2):851–858

Ferrara N, Houck KA, Jakeman LB, Winer J, Leung DW (1991) The vascular endothelial growth factor family of polypeptides. J Cell Biochem 47:211–218

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380(6573):439–442

Ferrara N (2004) Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 25:581–611

Ferrara N, Kerbel RS (2005) Angiogenesis as a therapeutic target. Nature 438:967–974

Gardner V, Madu C, Lu Y (2017) In Physiologic and Pathologic Angiogenesis. Signaling Mechanisms and Targeted Therapy In: Simionescu D (ed) Anti-VEGF Therapy in Cancer: A Double-Edged Sword, InTechOpen, London

Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N (1999) VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med 5(6):623–628

Ghaly AE, Kamal M & Correia LR (2005) Kinetic modeling of continuous submerged fermentation of cheese whey for single cell protein production. Bioresour Technol 96: 1143–1152

Gorski DH, Beckett MA, Jaskowiak NT, Calvin DP, Mauceri HJ, Salloum RM, Seetharam S, Koons A, Hari DM, Kufe DW, Weichselbaum RR (1999) Blockage of the vascular endothelial growth factor stress
response increases the antitumor effects of ionizing radiation. Cancer Res 59:3374–3378

Feng YM, Zhang BY, Zhang YS, Hiroshi F (1997) Secretory Expression of Porcine Insulin Precursor in Kluyveromyces lactis and Its Conversion into Human Insulin. Sheng wu hua xue yu Sheng wu wu li xue bao Acta Biochimica et Biophysica Sinica 29(2):129–134

Fiebich BL, Jager B, Schollmann C, Weindel K, Wilting J, Kochs G, Marme D, Hug H, Weich HA (1993) Synthesis and assembly of functionally active human vascular endothelial growth factor homodimers in insect cells. Eur J Biochem 211:19–26

Fleer R, Chen XJ, Amellal N, Yeh P, Fournier A, Guinet F, Gault N, Faucher D, Folliard F & Fukuhara H (1991a) High-level secretion of correctly processed recombinant human interleukin-1 beta in Kluyveromyces lactis. Gene 107:25–295

Frank S, Hübner G, Breier G, Longaker MT, Greenhalgh DG, Werner S (1995) Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing. J Biol Chem 270(21):12607–12613

Harju S, Fedosyuk H, Peterson KR (2004) Rapid isolation of yeast genomic DNA: Bust n’ Grab. BMC Biotechnol 4:8

Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA (2004) Vascular endothelial growth factor and angiogenesis. Pharmacol Rev 56:549–580

Holmes DI, Zachary I (2005) The vascular endothelial growth factor (VEGF) family: angiogenic factors in health and disease. Genome Biol 6(2):209

Hua Z, Liang X, Zhu D (1994) Expression and purification of a truncated macrophage colony stimulating factor in Kluyveromyces lactis. Biochem Mol Biol Int 34(2):419–427

Iijima K, Yoshikawa N, Connolly DT, Nakamura H (1993) Human mesangial cells and peripheral blood mononuclear cells produce vascular permeability factor. Kidney Int 44(5):959–966

Kang WK, Lee MH, Kim YH, Kim MY, Kim JY (2013) Enhanced secretion of biologically active, non-glycosylated VEGF from Saccharomyces cerevisiae. J Biotechnol 164(4):441–448

Keck RG, Berleau L, Harris R, Keyt BA (1997) Disulfide structure of the heparin binding domain in vascular endothelial growth factor: characterization of posttranslational modifications in VEGF. Arch Biochem Biophys 344(1):103–113

Keyt BA, Berleau LT, Nguyen HV, Chen H, Heinsohn H, Vandlen R, Ferrara N (1996) The carboxyl-terminal domain (111–165) of vascular endothelial growth factor is critical for its mitogenic potency. J Biol Chem 271:7788–7795
Kim HJ, Kim, HJ (2016) Yeast as an expression system for producing virus-like particles: What factors do we need to consider? Letters in Applied Microbiology 64:111–123

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lauer G, Sollberg S, Cole M, Krieg T, Eming SA (2002) Generation of a novel proteolysis resistant VEGF165 variant by a site-directed mutation at the plasmin sensitive cleavage site. FEBS Lett 531:309–313

Lee SS, Joo YS, Kim WU, Min DJ, Min JK, Park SH, Cho CS, Kim HY (2001) Vascular endothelial growth factor levels in the serum and synovial fluid of patients with rheumatoid arthritis. Clin Exp Rheumatol 19:321–324

Lee GY, Jung WW, Kang CS, Bang IS (2006) Expression and characterization of human vascular endothelial growth factor (VEGF165) in insect cells. Protein Expr Purif 46(2):503–509

Lee SB, Park JS, Lee S, Park J, Yu S, Kim H, Kim D, Byun TH, Baek K, Ahn YJ, Yoon J (2008) Overproduction of recombinant human VEGF (vascular endothelial growth factor) in Chinese hamster ovary cells. J Microbiol Biotechnol 18(1):183–187

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246:1306–1309

Li X, Eriksson U (2001) Novel VEGF family members: VEGF-B, VEGF-C and VEGF-D. Int J Biochem Cell Biol 33:421–426

Mohanraj D, Olson T, Ramakrishnan S (1995) Expression of biologically active human vascular endothelial growth factor in yeast. Growth Factors 12(1):17–27

Nguyen MT, Krupa M, Koo B-K, Song J-A, Vu TTT, Do BH, Nguyen AG, Seo T, Yoo J, Jeong B, Jin J, Lee KJ, Oh HB, Choe H (2016) Correction: Prokaryotic Soluble Overexpression and Purification of Human VEGF165 by Fusion to a Maltose Binding Protein Tag. PloS ONE 11(5):e0156296

Oishi H, Morimoto T, Watanabe Y, Tamai Y (1999) Purification and characterization of phospholipase B from Kluyveromyces lactis, and cloning of phospholipase B. Biosci Biotechnol Biochem 63:83–90

Peretz D, Gitay-Goren H, Safran M, Kimmel N, Gospodarowicz D, Neufeld G (1992) Glycosylation of vascular endothelial growth factor is not required for its mitogenic activity. Biochem Biophys Res Commun 182:1340–1347

Pizarro SA, Gunson J, Field MJ, Dinges R, Khoo S, Dalal M, Lee M, Kaleas KA, Moiseff K, Garnick S, Reilly DE, Laird MW, Schmelzer CH (2010) High-yield expression of human vascular endothelial growth factor VEGF165 in Escherichia coli and purification for therapeutic applications. Protein Expr Purif 72:184–193
Porro D, Bianchi MM, Brambilla L (1999) Replacement of a metabolic pathway for large-scale production of lactic acid from engineered yeasts. Appl Environ Microbiol 65:4211–4215

Reichardt LF, Tomaselli KJ (1991) Extracellular matrix molecules and their receptors: Functions in neural development. Annu Rev Neurosci 14:531–570

Riedel F, Gotte K, Goessler U, Sadick H, Hormann K (2004) Targeting chemotherapy-induced VEGF up-regulation by VEGF antisense oligonucleotides in HNSCC cell lines. Anticancer Res 24:2179–2183

Rocha TL, Paterson G, Crimmins K, Boyd A, Sawyer L, Fothergill-Gilmore LA (1996) Expression and secretion of recombinant ovine beta-lactuglobulin in Saccharomyces cerevisiae and Kluyveromyces lactis. Biochem J 313:927–932

Rocha S, Abrahao-Neto J, Cerdan M, Gombert A, Gonzalez-Siso M (2011) Heterologous expression of a thermophilic esterase in Kluyveromyces yeasts. Appl Microbiol Biotechnol 89(2):375–385

Rosa J, Colombo L, Alvim M, Avonce N, van Dijck P, Passos F (2013) Metabolic engineering of Kluyveromyces lactis for l-ascorbic acid (vitamin C) biosynthesis. Microb Cell Fact 12, 59

Scott PA, Gleadle JM, Bicknell R, Harris AL (1998) Role of the hypoxia sensing system, acidity and reproductive hormones in the variability of vascular endothelial growth factor induction in human breast carcinoma cell lines. Int J Cancer 75:706–712

Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219:983–985

Shibuya M (1995) Role of VEGF-Flt receptor system in normal and tumor angiogenesis. Adv Cancer Res 67:281–316

Shibuya M (2003) Vascular endothelial growth factor receptor-2: its unique signaling and specific ligand, VEGF-E. Cancer Sci 94:751–756

Shibuya M, Claesson-Welsh L (2006) Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. Exp Cell Res 312:549–560

Spohner SC, Schaum V, Quitmann H, Czermak P (2016) Kluyveromyces lactis: An emerging tool in biotechnology. Journal of Biotechnology 222:104–116

Sunderkötter C, Steinbrink K, Goebeler M, Bhardwaj R, Sorg C (1994) Macrophages and angiogenesis. J Leukoc Biol 55(3):410–422

Swennen D, Paul M, Vernis L, Beckerich J, Fournier A, Gaillardin C (2002) Secretion of active anti-Ras single-chain Fv antibody by the yeasts Yarrowia ipolytica and Kluyveromyces lactis. Microbiology 148(Pt 1):41–50
Takahashi H, Shibuya M (2005) The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. Clin Sci 109:227–241

Taktak-BenAmar A, Morjen M, Ben Mabrouk H, Abdelmaksoud-Dammak R, Guerfali M, Fourati-Masmoudi N, Marrakchi N, Gargouri A (2017) Expression, purification and functionality of bioactive recombinant human vascular endothelial growth factor VEGF165 in *E. coli*. AMB Express 7(1):33

Tammela T, Enholm B, Alitalo K, Paavonen K (2005) The biology of vascular endothelial growth factors. Cardiovasc Res 65:550–563

Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC, Abraham JA (1991) The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. J Biol Chem 266:11947–11954

Verheul HM, Hoekman K, Luykx-de Bakker S, Eekman CA, Folman CC, Broxterman HJ, Pinedo HM (1997) Platelet: transporter of vascular endothelial growth factor. Clin Cancer Res. 3(12 Pt 1):2187–2190

Wu X, Yin Z, Cao C, Huang L, Lu X, Liu J, Cui W (2004) Expression of human VEGF165 in silkworm (*Bombyx mori L.*) by using a recombinant baculovirus and its bioactivity assay. J Biotechnol 111(3):253–61

Xia Y-P, Li B, Hylton D, Detmar M, Yancopoulos GD, Rudge JS (2003) Transgenic delivery of VEGF to mouse skin leads to an inflammatory condition resembling human psoriasis. Blood 102:161–168

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**

Culture supernatant

Ammonium-sulphate precipitation

Ni-NTA chromatography

Dialysis

6xHis-tagged VEGF165
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.

![Western blot analysis](image)

**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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