

Construction of pPIC9 Recombinant Vector Containing Human Stem Cell Factor

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ARTICLE INFO

Article Type:

Research Article

Article History:

Received: 22 January 2013

Revised: 4 March 2013

Accepted: 12 March 2013

ePublished: 20 August 2013

Keywords:

Human SCF

Cloning

Expression

Pichia pastoris

ABSTRACT

Purpose: Various cytokine regulates hematopoiesis; they promote number of stages in stem cells biology such as proliferation, differentiation and endurance. Biological effects of SCF, as a hematopoietic cytokine; is triggered by binding to its ligand c-kit. Potential therapeutic applications of SCF include hematopoietic stem cell mobilization, ex vivo stem/progenitor cell expansion, gene therapy, and immunotherapy. In this study we tried to construct of pPIC9 recombinant vector containing human SCF. **Methods:** hSCF cDNA was amplified by PCR and both hSCF cDNA and pPIC9 as yeast expression vector (shuttle vector) digested by *EcoRI* and *XhoI* restriction enzymes. Subsequent the digestion reaction, ligation reaction was carried out. In order to verifying of pPIC9 recombinant vector containing hSCF, PCR and sequence analysis was performed. **Results:** The construction of recombinant expression vector of pPIC9 containing hSCF cDNA was confirmed by sequencing method successfully. **Conclusion:** rhSCF/pPIC9 vector can be transformed into the *Pichia pastoris* yeast as a eukaryotic host in order to produce human SCF at industrial scale.

Introduction

HEMATOPOIESIS is regulated by several cytokines that endorse the survival, proliferation, and differentiation of hematopoietic stem cells and progenitor cells.¹ Stem Cell Factor (SCF) plays an important role in hematopoiesis, spermatogenesis, and melanogenesis. Biological effects of SCF; as a hematopoietic cytokine; is triggered by binding to its ligand c-kit.²⁻⁴ The SCF gene is located on the Sl locus in mice⁴ and on chromosome 12q22-12q24 in humans.⁵⁻⁶ SCF can exist both as a transmembrane protein and a soluble protein⁷ (Figure 1, described in detail below). The both forms of SCF are produced by alternative splicing of the same RNA transcript and depending on inclusion or exclusion of exon 6, the proteolytic cleavage site can remain or not.⁸⁻⁹

Translation of mRNA including exon 6 contains proteolytic cleavage site and leads to production of the soluble form of human SCF (SCF248). Whereas the transmembrane form of SCF (SCF220) is produced by alternative spliced mRNA translation which excludes exon 6 that the cleavage occurs after Ala165. Gly residue which is replaced with amino acids 149-177 in SCF220.

The soluble form of SCF is glycosylated and circulates as a dimer. The SCF's soluble form has noticeable secondary structure which includes a helices and β sheets regions.¹⁰⁻¹³ The molecular weight of the soluble form of SCF is about 18.5 kDa. SCF contains four Cys residues which construct the two intramolecular bonds

Cys4-Cys89 and Cys43-Cys138.¹⁴⁻¹⁵ Deletion of the area containing Cys138 in carboxyterminal region reduces biologic activity of the soluble SCF. This concept recommends that the Cys43-Cys138 disulfide bond might be essential for its full biologic activity.¹⁶ Subsequent studies show that both intramolecular disulfide pairs are important to retain SCF entire biologic activity.¹⁷

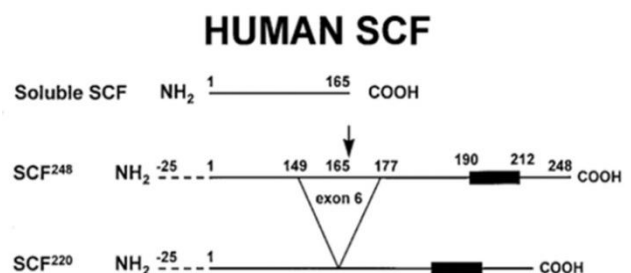


Figure 1. Soluble and transmembrane forms of human stem cell factor are shown. The arrow points to the primary proteolytic cleavage site of SCF₂₄₈ in exon 6. The transmembrane form of SCF, SCF₂₂₀ lacks the primary proteolytic cleavage site in exon 6. Dotted lines show the 25 amino acid signal sequence and dark box indicates the hydrophobic transmembrane domain.

SCF has a great role in acceleration of hematopoietic stem cells entry into the cell cycle.¹⁸ Colony forming unit- spleen CFU-S survival *in vitro* needs the presence

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of SCF, and when SCF accompanies with IL-3 *in vitro*, the production of CFU-S increases significantly along a two-week period.¹⁹

The mast cell deficiency in W/W^v and Sl/Sl^d mice indicated that SCF might be necessary for mast cell production. Mast cells survival, proliferation, and maturation are promoted by SCF *in vitro*. *In vitro* proliferation and differentiation of pro-mastocyte, the earliest committed mast cell progenitor, occurs in the presence of both SCF and IL-3.²⁰

In according to mast cell studies,^{7,21-23} hematopoietic cell lines studies^{24,25} and of normal hematopoietic cells studies,²⁵ we can conclude that SCF can regulate the adhesive properties of hematopoietic cells.

CD34⁺ marrow cells adherence to fibronectin is enhanced by SCF Exposure, and hematopoietic

progenitor cells adhesion to stromal cells decreases in W/W^v mice.²⁶

Sensitivity to radiotherapy changes in the presence of SCF. Sl and W mutations increases radiosensitivity in mice²⁷ it might be due to the capability of SCF to suppress apoptosis and promote cell cycle progression.^{4,28}

According to the properties of SCF, this protein can be used in clinical applications. Thus production of human Stem Cell Factor as a recombinant protein is a necessity in our country. We tried to construct recombinant shuttle vector of pPIC9 contains human stem cell factor gene. This vector can be used for transformation into *pichia pastoris* yeast. The Figure 2 shows the map of pPIC9.

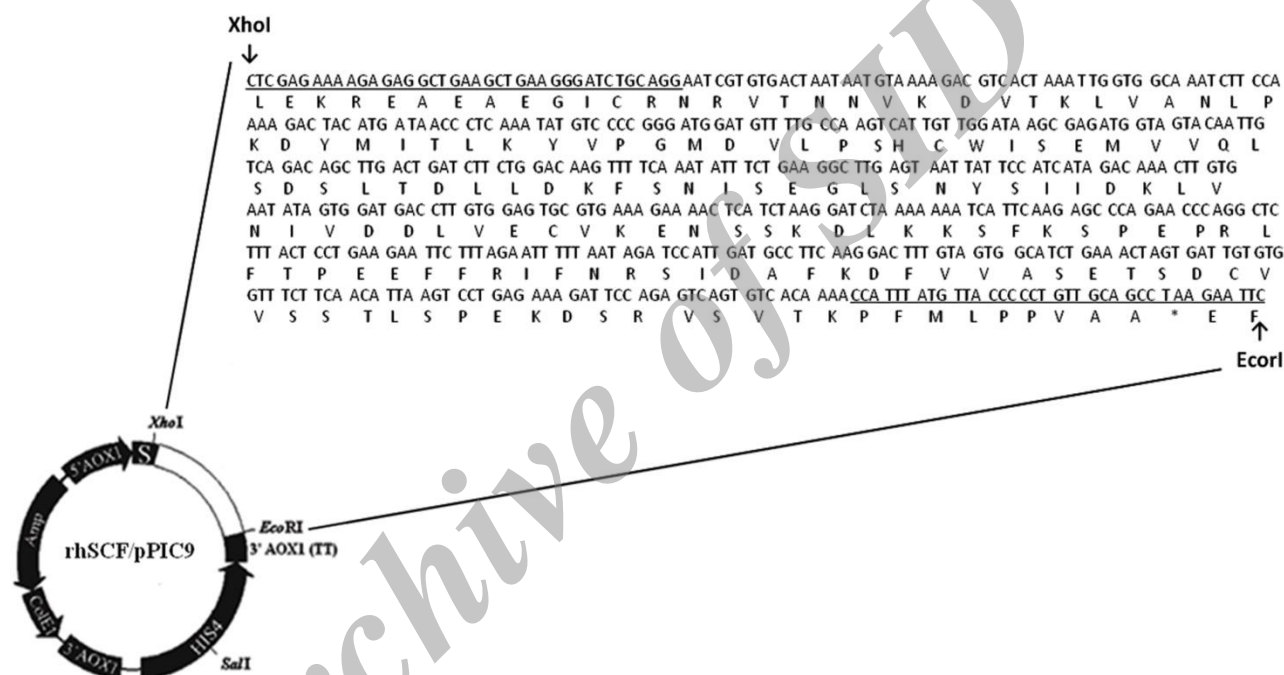


Figure 2. Expression vector used for production of recombinant *hSCF* in *Pichia pastoris*. The *hSCF* cDNA nucleotide sequence is shown in details and sequences belong to primers which used in specific amplification of cDNA. Sequences found in the vector: 5' AOX1-alcohol oxidase promoter, 3' AOX1(TT)- Transcriptional termination sequence, using 3' AOX1- sequences for direct integration into the yeast genome, HIS4 -histidyl dehydrogenase codifying gene, S-secretion signal sequence, ColE1-Escherichia coli replication origin, Amp -ampicillin resistance gene in Escherichia coli.

Materials and Methods

PCR Amplification of *hSCF* cDNA

The SCF cDNA sequence (protein coding nucleotides) was amplified by PCR, using specific primers), corresponding to the N- and C-terminal amino acid sequence of SCF. The human SCF cDNA sequence was derived from NCBI gene bank (Homo sapiens KIT ligand (KITLG), transcript variant b, mRNA. ACCESSION # NM_000899). Recognition sites sequence of XhoI and EcoR I restriction enzymes were added to 5' ends of reverse and forward primers to introduce Xho I site at the 5' and EcoR I site (Italics and bold) at the 3' end of the PCR products. We included the sequence between Xho I site and SnaBI site (underlined) in the 5' site of the SCF-fwd primer

after the XhoI recognition site sequence. These 24 nucleotides between Xho I site and SnaBI site which encodes the KEX2 and STE13 cleavage sites must be recreated in order for efficient cleavage of the fusion protein to occur. Meanwhile the stop codon (TAA) was included after the EcoR I site in reverse primer. The sequences of the primers were 5'-ATCTCGAGAAAAGAGAGGCTGAAGCTGAAGG GATCTGCAGG-3' and 5'-AATGAATTCTTAGGCTGCAACAGGGGTAACA TAAATGG-3'.

A 498 bp fragment including whole coding sequence of SCF gene was amplified with these primers by PCR in a final volume 50 μ l. The final concentration of materials for PCR was as follows: Primers: 0.4 μ M,

dNTP: 200 μ M and Mg²⁺: 1.5mM. PFU polymerase was used for amplification. Reaction was performed at an initial denaturation at 94 °C for 3 min followed by 30 cycles amplification, each cycle including denaturation for 30 sec, annealing at 68 °C for 1 min and extension at 72 °C for 1 min with an extra final 10 min incubation at 72 °C to complete all extensions.

Cloning of hSCF cDNA into pPIC9 Expression Vector

pPIC9 Vector and PCR product were double digested by *Xho*I/*Eco*R1 enzymes at 37 °C over night (Fermentas) separately. Final volume of reaction was 40 μ l and tango buffer was used as buffer in the reaction. Following electrophoresis of digested products, extraction of digested vector and insert from gel agarose was performed using QIAGEN kit. Ligation was performed with T4 DNA Ligase enzyme and 100ng of vector with the ratio 1:3 of insert to vector. Following ligation, transformation to the DH5- α strain was performed by CaCl₂ method. Transformant cells were plated on LB Agar containing Ampicillin (50 μ l/ml) and incubated overnight at 37 °C. Recombinant plasmids were extracted from several clones by miniprep kit (Fermentas).

Verifying of Recombinant Vector by PCR and Sequencing

PCR was performed at the same conditions on extracted plasmids with SCF-Fwd and SCF-Rev primers. PCR product were analysed by sequencing.

Results

The SCF cDNA was amplified by PCR. A single band of the expected size (498 bp) corresponding to human SCF cDNA was detected by agarose gel electrophoresis (Figure 3).

Double digestion of pPIC9 vector was performed with *Xho*I/*Eco*R1 restriction enzymes. Digested products were resolved on agarose gel using electrophoresis and gel picture is presented in Figure 4. Following double digestion of vector by *Xho*I/*Eco*R, a small fragment of pPIC9 vector including 40 bp is separated which was electrophoresed out of gel and not observed on the gel. The bound A belongs to undigested vector and bounds B and C belong to digested vectors (Figure 4).

Following *Xho*I/*Eco*R1 digestion and purification of both vector and PCR product, the PCR product was ligated into the corresponding *Eco*R I and *Xho*I sites within the multi-cloning site (MCS) of pPIC9 plasmid. Subsequent the ligation reaction, bacterial transformation and Amp selection on LB agar plates, a few numbers of clones were obtained. A total of 10 bacterial clones were screened by PCR using the SCF-Fwd and SCF-Rev primers. These primers yielded cloned PCR products containing the 498 bp PCR product representing SCF cDNA (Figure 5).

Sequence analysis of the 498 bp cloned PCR products confirmed SCF cDNA (Figure 6).

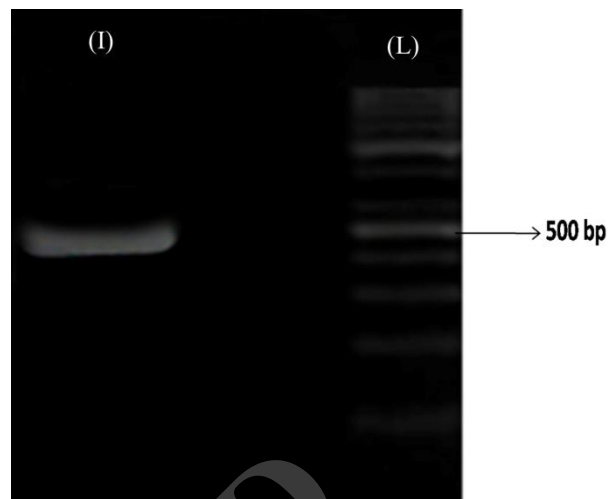


Figure 3. Agarose gel electrophoresis of PCR products of SCF cDNA. Lane of (I) corresponds to PCR Product of human SCF cDNA. Lane (L) corresponds to 100bp Ladder (Fermentas).

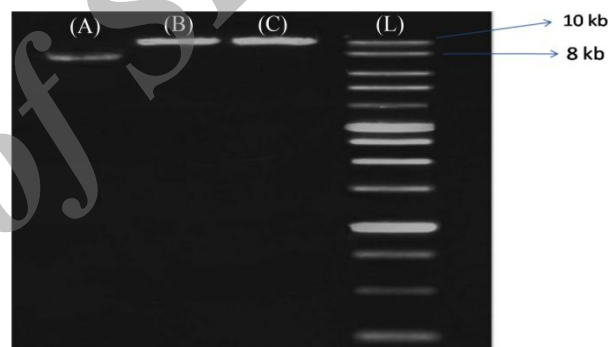


Figure 4. Double digestion of pPIC9 vector by *Xho*I/*Eco*R. bound A correspond to undigested vector. bound s B and C correspond to double digested pPIC9 vector. Lane L correspond to 10 Kbp ladder.

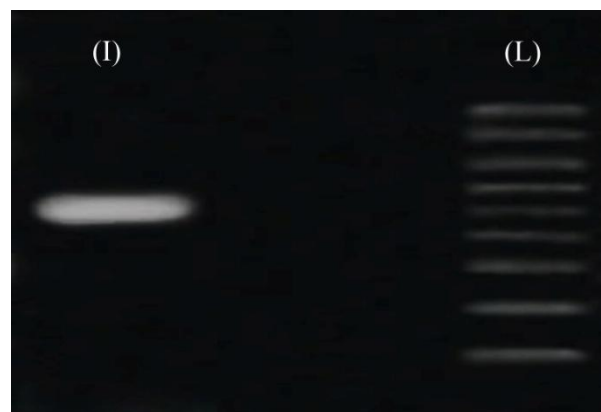


Figure 5. PCR screening of cloned SCF cDNA PCR products. The clones on Ampicillin plates were picked and screened by PCR. Lane L corresponds to 100 bp ladder (Fermentas). Bound I represent bacterial clone containing the SCF cDNA PCR product.

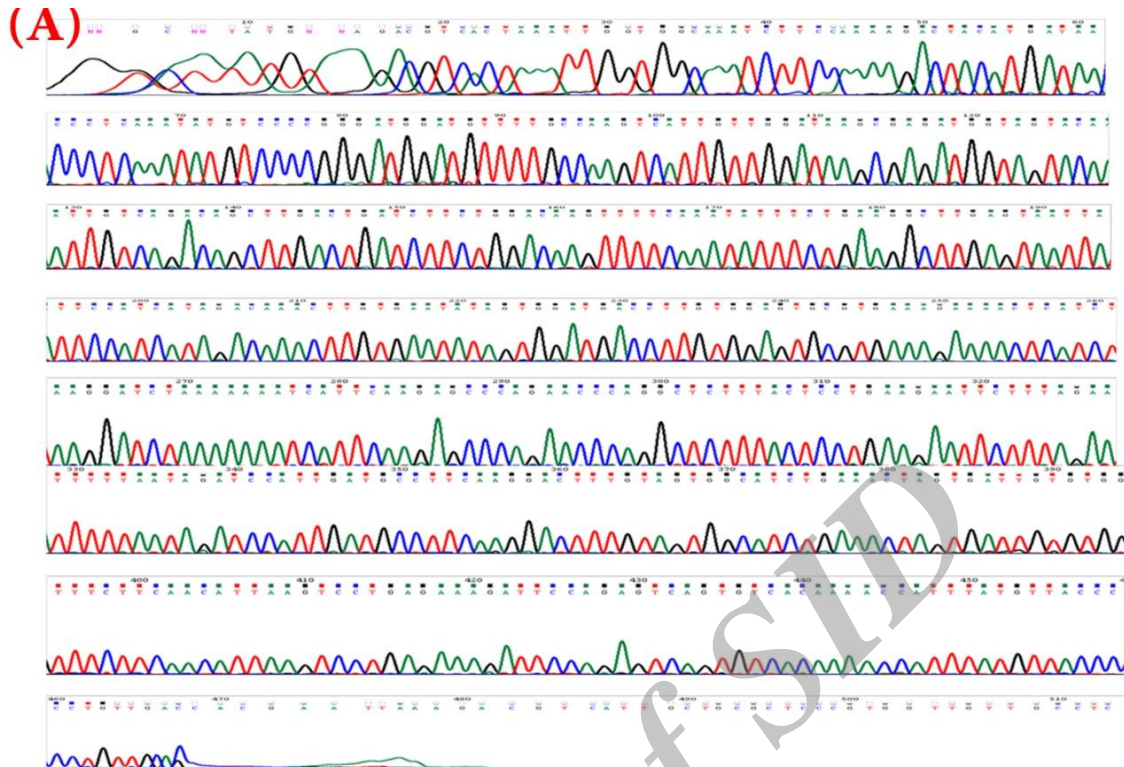


Figure 6. Sequencing analysis of recombinant vector. A) Chromatogram of sequencing result related to SCF cDNA. B) Blast analysis of the sequencing result. The results reveal that 498 bp bond which obtained from PCR reaction which belongs to human SCF.

This approach allowed heterologous gene insertion between the 5' AOX1 promoter sequence and the transcription termination sequence. The resultant

plasmid (rhSCF/pPIC9), shown in Figure 1, was transformed into *Escherichia coli*, enabling rhSCF expression plasmid propagation.

Discussion

The survival, differentiation, and mobilization of numerous cell types (myeloid, erythroid, megakaryocytic, lymphoid, germ cell, and melanocyte progenitors) are promoted by SCF.^{29,30}

SCF is widely used in stem cell research area and purchasing this recombinant protein is costly and time-consuming in Iran. Considering the cost and problem in accessibility of this product, clears the importance of indigenizing production of rhSCF.

The occurrence of multiple disulfide bridges in the native SCF protein (5) prompted us to use the *Pichia pastoris* eukaryotic expression system, since it is known that disulfide bridge formation of eukaryotic proteins expressed in prokaryotes is often erratic, leading to improper folding and tertiary structure destabilization.

For expression of SCF gene under the transcriptional control of AOX1 promoter, a 498 bp *XhoI/EcoR I* fragment composed of a 498-bp region beginning immediately 5' of methionine initiator ATG of SCF was generated by PCR.

In designing primers for cloning SCF coding gene in pPIC9 vector, we attempted to clone the SCF ORF under control of AOX1 promoter with α -factor secretion signal sequence in downstream of the AOX1 promoter. Recent evidences suggest that the α -factor secretion signal sequence might be modified to include KEX2-like processing sites for efficient cleavage to occur.³¹ Choosing *Xho I* restriction site in the 5'-end allowed in-frame cloning into the α -factor secretion signal of pPIC9 expression vector and a sequence encoding the KEX2 and STE13 cleavage sites comprising 24 nucleotides was placed ahead of the mature SCF cDNA. The reverse primer was designed based on the C-terminal amino acid sequence of SCF, a stop codon, and an *EcoRI* restriction site in the 3'-end.

Using these primers, the 498-bp fragment encoding hSCF was cloned in pPIC9 inframe to the α -factor secretion signal, downstream of the alcohol oxidase promoter. The resultant plasmid (rhSCF/pPIC9) was transformed into *E. coli* DH5 α , purified, and analyzed by sequencing, confirming the presence of rhSCF/pPIC9 expression plasmid.

Acknowledgements

Authors are highly thankful to stem cell research center for financial support.

Conflict of Interest

The authors report no conflicts of interest.

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