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Construction of pPIC9 Recombinant Vector Containing Human Stem Cell Factor

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ABSTRACT

Purpose: Various cytokine regulates hematopoesis; 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, exvivo 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 *EcoR I* and *Xho I* 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 *Picha 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 mice[4] and on chromosome 12q22-12q24 in humans.[5-6] SCF can exist both as a transmembrane protein and a soluble protein[7] (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 exone 6 that the cleavage occurs after Ala165. Gly residue which is replace 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]

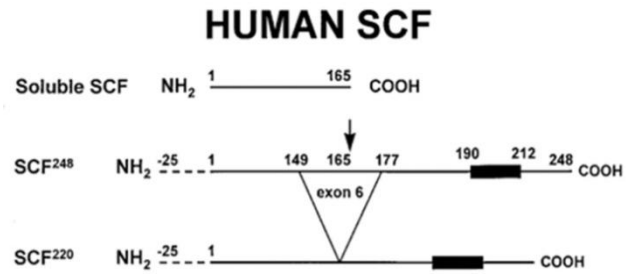

Figure 1. Soluble and transmembrane forms of human stem cell factor are shown. The arrow points to the primary proteolytic cleavage site of SCF_{248} in exon 6. The transmembrane form of SCF, SCF_{220} lacks the primary proteolytic cleavage site in exon 6. Dotted lines show the 25 amino acid signal sequence 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/Wv and Sl/Sld 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/Wv mice.[26]

Sensitivity to radiotherapy changes in the presence of SCF. Sl and W mutations increases radiosensitivity in mice[27] 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.

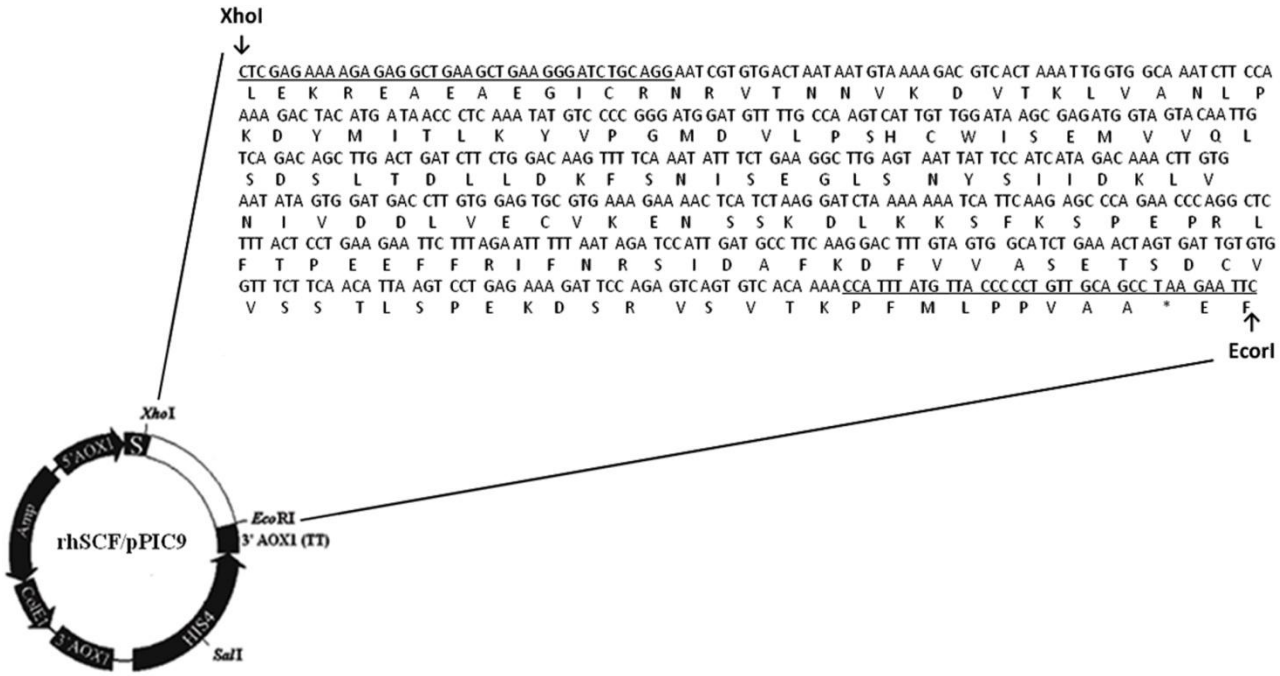


Figure 2. Expression vector used for production of recombinant *hSCF* in *Pichia pastoris*. The *hSCF* cDNA nucleotide sequence in shown in details and sequences belong to primers which used in specific amplification of cDNA. Sequences found in the vector: 5' AOX1-alcohol oxidase promoter, 3'AOX1(TT)- Transcriptional termination sequence,using 3' AOX1– sequences for direct integration into the yeast genome, HIS4 –histidiol 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 Xho1 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' AATGAATTCTTAGGCTGCAACAGGGGGTAACA TAAATGG-3'.

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

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

Cloning of hSCF cDNA into pPIC9 Expression Vector

pPIC9 Vector and PCR product were double digested by *Xho1*/*EcoR1* enzymes at 37 ºC over night (Fermentas) separately. Final volume of reaction was 40 µl 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 QIAGENE 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 Ampicilin (50µl/ml) and incubated overnight at 37 ºC. 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 *Xho1*/*EcoR1* 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 Xho1/EcoR, a small fragment of pPIC9 vector including 40 bp is separated which was electrophorsed 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/EcoR I* digestion and purification of both vector and PCR product, the PCR product was ligated into the corresponding *EcoR* 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).

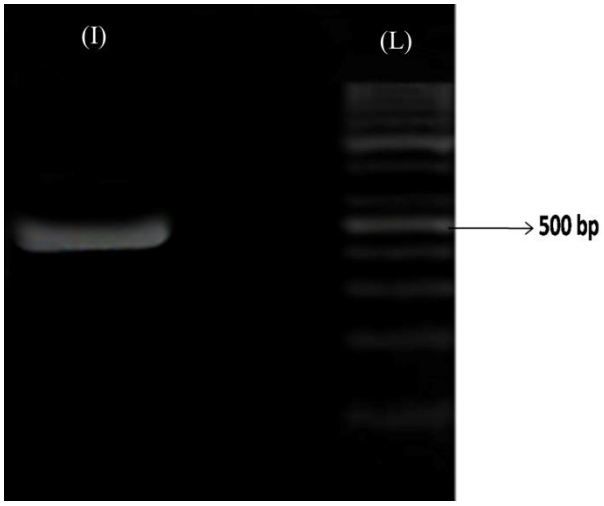


Figure 3. Agarose gel electrophoresis of PCR products of SCF cDNA. Lane of (I) corresponds to PCR Product of human SCF cDNA. Lane (L) corresponds to 100bp Ladder (Fermentas).

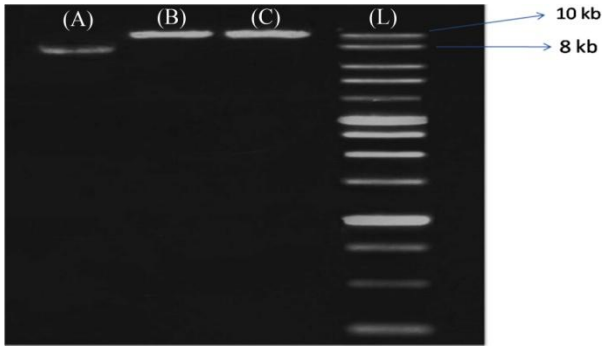


Figure 4. Double digestion of pPIC9 vector by Xho1/EcoR. bound A correspond to undigested vector. bound s B and C correspond to double digested pPIC9 vector. Lane L correspond to 10 Kbp ladder.

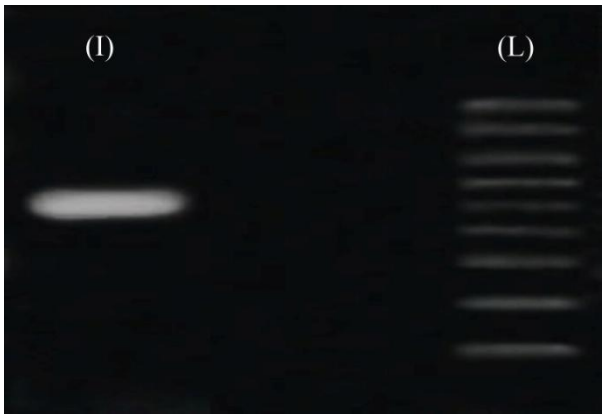
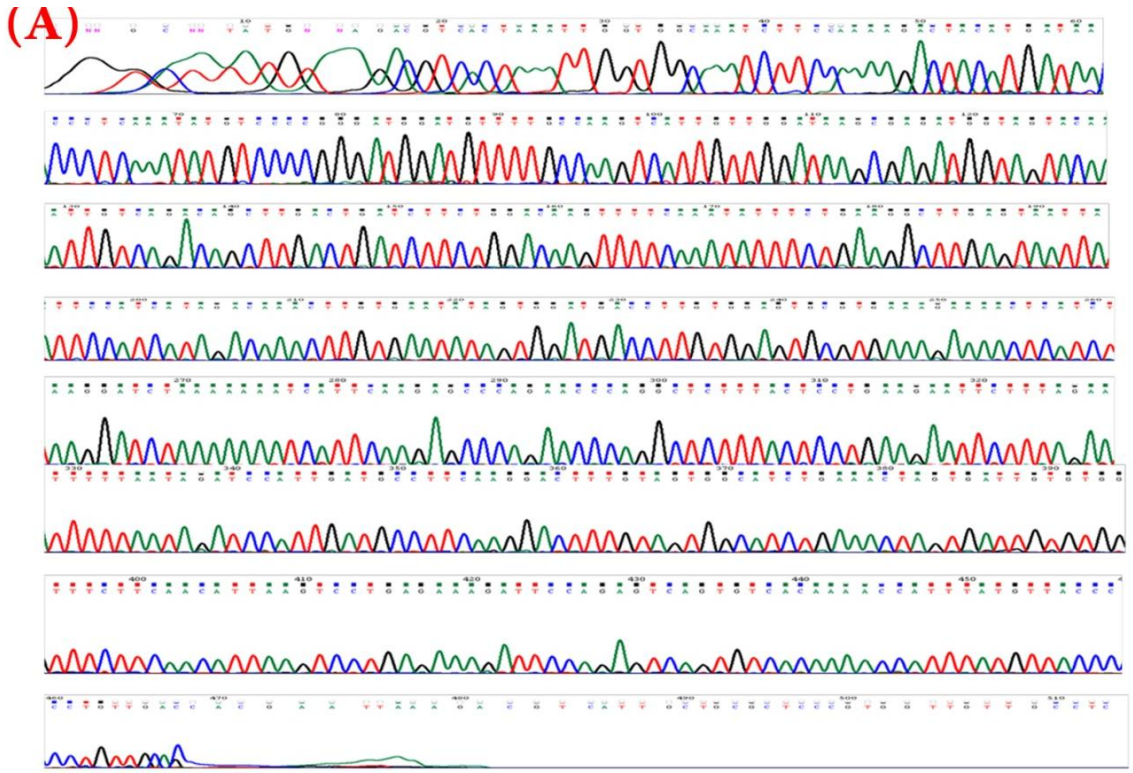


Figure 5. PCR screening of cloned SCF cDNA PCR products. The clones on Ampicillin plates were picked and screened by PCR. Lane L corresponds to 100 bp ladder (Fermentas). Bound I represent bacterial clone containing the SCF cDNA PCR product.



(B)

ref|NM_000899.4| UEGM Homo sapiens KIT ligand (KITLG), transcript variant b, mRNA
Length=5460

Score = 835 bits (452), Expect = 0.0
Identities = 452/452 (100%), Gaps = 0/452 (0%)
Strand=Plus/Plus

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Query  15   AGACGTCACTAAATTGGTGGCAAATCTTCCAAAAGACTACATGATAACCCTCAAATATGT  74
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  309  AGACGTCACTAAATTGGTGGCAAATCTTCCAAAAGACTACATGATAACCCTCAAATATGT  368

Query  75   CCCCGGGATGGATGTTTTGCCAAGTCATTGTTGGATAAGCGAGATGGTAGTACAATTGTC  134
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  369  CCCCGGGATGGATGTTTTGCCAAGTCATTGTTGGATAAGCGAGATGGTAGTACAATTGTC  428

Query  135  AGACAGCTTGACTGATCTTCTGGACAAGTTTTCAAATATTTCTGAAGGCTTGAGTAATTA  194
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  429  AGACAGCTTGACTGATCTTCTGGACAAGTTTTCAAATATTTCTGAAGGCTTGAGTAATTA  488

Query  195  TTCCATCATAGACAAACTTGTGAATATAGTGGATGACCTTGTGGAGTGCGTGAAAGAAAA  254
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  489  TTCCATCATAGACAAACTTGTGAATATAGTGGATGACCTTGTGGAGTGCGTGAAAGAAAA  548

Query  255  CTCATCTAAGGATCTAAAAAAATCATTCAAGAGCCCAGAACCCAGGCTCTTTACTCCTGA  314
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  549  CTCATCTAAGGATCTAAAAAAATCATTCAAGAGCCCAGAACCCAGGCTCTTTACTCCTGA  608

Query  315  AGAATTCTTTAGAATTTTTAATAGATCCATTGATGCCTTCAAGGACTTTGTAGTGGCATC  374
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  609  AGAATTCTTTAGAATTTTTAATAGATCCATTGATGCCTTCAAGGACTTTGTAGTGGCATC  668

Query  375  TGAAACTAGTGATTGTGTGGTTTCTTCAACATTAAGTCCTGAGAAAGATTCCAGAGTCAG  434
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  669  TGAAACTAGTGATTGTGTGGTTTCTTCAACATTAAGTCCTGAGAAAGATTCCAGAGTCAG  728

Query  435  TGTCACAAAACCATTTATGTTACCCCCTGTTG  466
           ||||||||||||||||||||||||||||||||
Sbjct  729  TGTCACAAAACCATTTATGTTACCCCCTGTTG  760
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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 a-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 a-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 DH5α, purified, and analyzed by sequencing, confirming the presence of rhSCF/pPIC9 expression plasmid.

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Conflict of Interest

The authors report no conflicts of interest.

References

1. Metcalf D. Hematopoietic regulators: redundancy or subtlety? *Blood* 1993;82(12):3515-23.
2. Williams DE, Eisenman J, Baird A, Rauch C, Ness KV, March CJ, et al. Identification of a ligand for the c-kit protooncogene. *Cell* 1990;63(1):167-74.
3. Flanagan JG, Leder P. The kit ligand: a cell surface molecule altered in steel mutant fibroblasts. *Cell* 1990;63(1):185-94.
4. Zsebo KM, Williams DA, Geissler EN, Broudy VC, Martin, FH, Atkins HL, et al. Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell* 1990;63:213.
5. Anderson DM, Williams DE, Tushinski R, Gimpel S, Eisenman J, Cannizzaro LA, et al. Alternate splicing of mRNAs encoding human mast cell growth factor and localization of the gene to chromosome 12q22-q24. *Cell Growth Differ* 1991;2(8):373-8.
6. Geissler EN, Liao M, Brook JD, Martin FH, Zsebo KM, Housman DE, et al. Stem cell factor (SCF), a novel hematopoietic growth factor and ligand for c-kit tyrosine kinase receptor, map on human chromosome 12 between 12q14.3 and 12qter. *Somat Cell Mol Genet* 1991;17(2):207-14.
7. Flanagan JG, Chan DC, Leder P. Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the Sld mutant. *Cell* 1991;64(5):1025-35.
8. Huang EJ, Nocka KH, Buck J, Besmer P. Differential expression and processing of two cell associated forms of the kit-ligand. KL-1 and KL-2. *Mol Biol Cell* 1992;3(3):349-62.
9. Anderson DM, Lyman SD, Baird A, Wignall JM, Eisenman J, Rauch C, et al. Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. *Cell* 1990;63(1):235-43.
10. Toksoz D, Zsebo KM, Smith KA, Hu S, Brankow D, Suggs SV, et al. Support of human hematopoiesis in long-term bone marrow cultures by murine stromal cells selectively expressing the membrane-bound and secreted forms of the human homolog of the steel gene product, stem cell factor. *Proc Natl Acad Sci USA* 1992;89(16):7350-4.
11. Huang EJ, Manova K, Packer AI, Sanchez S, Bachvarova RF, Besmer P. The murine steel panda mutation affects kit ligand expression and growth of early ovarian follicles. *Dev Biol* 1993;157(1):100-9.
12. Huang E, Nocka K, Beier DR, Chu TY, Buck J, Lahm HW, et al. The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the c-kit receptor, the gene product of the W locus. *Cell* 1990;63(1):225-33.
13. Lu HS, Clogston CL, Wypych J, Fausset PR, Lauren S, Mendiaz EA, et al. Amino acid sequence and posttranslational modification of stem cell factor isolated from Buffalorat liver cell-conditioned medium. *J Biol Chem* 1991;266(13):8102-7.
14. Zsebo KM, Wypych J, McNiece IK, Lu HS, Smith KA, Karkare SB, et al. Identification, purification, and biological characterization of hematopoietic

stem cell factor from buffalo rat liver-conditioned medium. *Cell* 1990;63(1):195-201.
15. Langley KE, Wypych J, Mendiaz EA, Clogston CL, Parker VP, Farrar DH, et al. Purification and characterization of soluble forms of human and rat stem cell factor recombinantly expressed by Escherichia coli and by Chinese hamster ovary cells. *Arch Biochem Biophys* 1992;295(1):21-8
16. Nishikawa M, Tojo A, Ikebuchi K, Katayama K, Fujii N, Ozawa K, et al. Deletion mutagenesis of stem cell factor defines the c-terminal sequences essential for its biological activity. *Biochem Biophys Res Commun* 1992;188(1):292-7.
17. Jones MD, Narhi LO, Chang WC, Lu HS. Refolding and oxidation of recombinant human stem cell factor produced in Escherichia coli. *J Biol Chem* 1996;271(19):11301-8.
18. Leary AG, Zeng HQ, Clark SC, Ogawa M. Growth factor requirements for survival in G0 and entry into the cell cycle of primitive human hemopoietic progenitors. *Proc Natl Acad Sci USA* 1992;89(9):4013-7.
19. De Vries P, Brasel KA, Eisenman JR, Alpert AR, Williams DE. The effect of recombinant mast cell growth factor on purified murine hematopoietic stem cells. *J Exp Med* 1991;173(5):1205-11.
20. Rodewald HR, Dessing M, Dvorak AM, Galli SJ. Identification of a committed precursor for the mast cell lineage. *Science* 1996;271(5250):818-22.
21. Dastych J, Metcalfe DD. Stem cell factor induces mast cell adhesion to fibronectin. *J Immunol* 1994;152(1):213-9.
22. Kinashi T, Springer TA. Steel factor and c-kit regulate cell matrix adhesion. *Blood* 1994;83(4):1033-8.
23. Kaneko Y, Takenawa J, Yoshida O, Fujita K, Sugimoto K, Nakayama H, et al. Adhesion of mouse mast cells to fibroblasts: adverse effects of steel (Sl) mutation. *J Cell Physiol* 1991;147(2):224-30.
24. Kovach NL, Lin N, Yednock T, Harlan JM, Broudy VC. Stem cell factor modulates avidity of a4b1 and a5b1 integrins expressed on hematopoietic cell lines. *Blood* 1995;85(1):159-67.
25. Levesque JP, Leavesley DI, Niutta S, Vadas M, Simmons PJ. Cytokines increase human hemopoietic cell adhesiveness by activation of very late antigen (VLA)-4 and VLA-5 integrins. *J Exp Med* 1995;181(5):1805-15.
26. Kodama H, Nose M, Niida S, Nishikawa S, Nishikawa S. Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells. *Exp Hematol* 1994;22(10):979-84.
27. Russell ES, Bernstein SE, McFarland EC, Modeen WR. The cellular basis of differential radiosensitivity of normal and geneti cally anemic mice. *Radiat Res* 1963;20:677-94.
28. Geissler EN, McFarland EC, Russell ES. Analysis of pleiotropism at the dominant white-spotting (W) locus of the house mouse: A description of ten new W alleles. *Genetics* 1981;97(2):337-61.
29. Bashamboo A, Taylor AH, Samuel K, Panthier JJ, Whetton AD, Forrester LM. The survival of differentiating embryonic stem cells is dependent on the SCF-KIT pathway. *J Cell Sci* 2006;119(Pt 15):3039-46.
30. Ashman LK. The biology of stem cell factor and its receptor C-kit. *Int J Biochem Cell Biol* 1999;31(10):1037-51.
31. Laroche Y, Storme V, De Meutter J, Messens J, Lauwereys M. High-level secretion and very efficient isotopic labeling of tick anticoagulant peptide (TAP) expressed in the methylotrophic yeast, Pichia pastoris. *Biotechnol (N Y)* 1994;12(11):1119-24.

