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STEM CELL FACTOR

~~This is a continuation-in-part application of~~ Ser. No. 573,616 filed ~~August~~ 24, 1990 which ~~is a~~ continuation-in-part application of Ser. ~~No.~~ 537,198 filed June ~~11~~, 1990 which is a continuation-in-part application of Ser. No. 422,383 filed October 16, 1989 ~~hereby incorporated by reference~~.

The present invention relates in general to novel factors which stimulate primitive progenitor cells including early hematopoietic progenitor cells, and to DNA sequences encoding such factors. In particular, the invention relates to these novel factors, to fragments and polypeptide analogs thereof and to DNA sequences encoding the same.

Background of the Invention

The human blood-forming (hematopoietic) system is comprised of a variety of white blood cells (including neutrophils, macrophages, basophils, mast cells, eosinophils, T and B cells), red blood cells (erythrocytes) and clot-forming cells (megakaryocytes, platelets).

It is believed that small amounts of certain hematopoietic growth factors account for the differentiation of a small number of "stem cells" into a variety of blood cell progenitors for the tremendous proliferation of those cells, and for the ultimate differentiation of mature blood cells from those lines. The hematopoietic regenerative system functions well under normal conditions. However, when stressed by chemotherapy, radiation, or natural myelodysplastic disorders, a resulting period during which patients are seriously leukopenic, anemic, or thrombocytopenic occurs. The development and the use of hematopoietic

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growth factors accelerates bone marrow regeneration during this dangerous phase.

In certain viral induced disorders, such as acquired autoimmune deficiency (AIDS) blood elements such as T cells may be specifically destroyed. Augmentation of T cell production may be therapeutic in such cases.

Because the hematopoietic growth factors are present in extremely small amounts, the detection and identification of these factors has relied upon an array of assays which as yet only distinguish among the different factors on the basis of stimulative effects on cultured cells under artificial conditions.

The application of recombinant genetic techniques has clarified the understanding of the biological activities of individual growth factors. For example, the amino acid and DNA sequences for human erythropoietin (EPO), which stimulates the production of erythrocytes, have been obtained. (See, Lin, U. S. Patent 4,703,008, hereby incorporated by reference). Recombinant methods have also been applied to the isolation of cDNA for a human granulocyte colony-stimulating factor, G-CSF (See, Souza, U. S. Patent 4,810,643, hereby incorporated by reference), and human granulocyte-macrophage colony stimulating factor (GM-CSF) [Lee, et al., Proc. Natl. Acad. Sci. USA, 82, 4360-4364 (1985); Wong, et al., Science, 228, 810-814 (1985)], murine G- and GM-CSF [Yokota, et al., Proc. Natl. Acad. Sci. (USA), 81, 1070 (1984); Fung, et al., Nature, 307, 233 (1984); Gough, et al., Nature, 309, 763 (1984)], and human macrophage colony-stimulating factor (CSF-1) [Kawasaki, et al., Science, 230, 291 (1985)].

The High Proliferative Potential Colony Forming Cell (HPP-CFC) assay system tests for the action of factors on early hematopoietic progenitors [Zont, J. Exp. Med., 159, 679-690 (1984)]. A number of reports

exist in the literature for factors which are active in the HPP-CFC assay. The sources of these factors are indicated in Table 1. The most well characterized factors are discussed below.

An activity in human spleen conditioned medium has been termed synergistic factor (SF). Several human tissues and human and mouse cell lines produce an SF, referred to as SF-1, which synergizes with CSF-1 to stimulate the earliest HPP-CFC. SF-1 has been reported in media conditioned by human spleen cells, human placental cells, 5637 cells (a bladder carcinoma cell line), and EMT-6 cells (a mouse mammary carcinoma cell line). The identity of SF-1 has yet to be determined. Initial reports demonstrate overlapping activities of interleukin-1 with SF-1 from cell line 5637 [Zsebo et al., Blood, 71, 962-968 (1988)]. However, additional reports have demonstrated that the combination of interleukin-1 (IL-1) plus CSF-1 cannot stimulate the same colony formation as can be obtained with CSF-1 plus partially purified preparations of 5637 conditioned media [McNiece, Blood, 73, 919 (1989)].

The synergistic factor present in pregnant mouse uterus extract is CSF-1. WEHI-3 cells (murine myelomonocytic leukemia cell line) produce a synergistic factor which appears to be identical to IL-3. Both CSF-1 and IL-3 stimulate hematopoietic progenitors which are more mature than the target of SF-1.

Another class of synergistic factor has been shown to be present in conditioned media from TC-1 cells (bone marrow-derived stromal cells). This cell line produces a factor which stimulates both early myeloid and lymphoid cell types. It has been termed hemolymphopoietic growth factor 1 (HLGF-1). It has an apparent molecular weight of 120,000 [McNiece et al., Exp. Hematol., 16, 383 (1988)].

Of the known interleukins and CSFs, IL-1, IL-3, and CSF-1 have been identified as possessing activity in the HPP-CFC assay. The other sources of synergistic activity mentioned in Table 1 have not been structurally identified. Based on the polypeptide sequence and biological activity profile, the present invention relates to a molecule which is distinct from IL-1, IL-3, CSF-1 and SF-1.

Table 1
Preparations Containing Factors Active in the HPP-CFC Assay

Source [1]	Reference
Human Spleen CM	[Kriegler, Blood, 60, 503(1982)]
Mouse Spleen CM	[Bradley, Exp. Hematol. Today Baum, ed., 285 (1980)]
Rat Spleen CM	[Bradley, supra, (1980)]
Mouse lung CM	[Bradley, supra, (1980)]
Human Placental CM	[Kriegler, supra (1982)]
Pregnant Mouse Uterus	[Bradley, supra (1980)]
GTC-C CM	[Bradley, supra (1980)]
RH3 CM	[Bradley, supra (1980)]
PHA PBL	[Bradley, supra (1980)]
WEHI-3B CM	[McNiece, Cell Biol. Int. Rep., 6, 243(1982)]
EMT-6 CM	[McNiece, Exp. Hematol., 15, 854 (1987)]
L- Cell CM	[Kriegler, Exp. Hematol., 12, 844 (1984)]
5637 CM	[Stanley, Cell, 45, 667 (1986)]
TC-1 CM	[Song, Blood, 66, 273 (1985)]

[1] CM= Conditioned media.

When administered parenterally, proteins are often cleared rapidly from the circulation and may

therefore elicit relatively short-lived pharmacological activity. Consequently, frequent injections of relatively large doses of bioactive proteins may be required to sustain therapeutic efficacy. Proteins modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone or polyproline are known to exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified proteins [Abuchowski et al., In: "Enzymes as Drugs", Holcenberg et al., eds. Wiley-Interscience, New York, NY, 367-383 (1981), Newmark et al., J. Appl. Biochem. 4:185-189 (1982), and Katre et al., Proc. Natl. Acad. Sci. USA 84, 1487-1491 (1987)]. Such modifications may also increase the protein's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the protein, and greatly reduce the immunogenicity and antigenicity of the protein. As a result, the desired in vivo biological activity may be achieved by the administration of such polymer-protein adducts less frequently or in lower doses than with the unmodified protein.

Attachment of polyethylene glycol (PEG) to proteins is particularly useful because PEG has very low toxicity in mammals [Carpenter et al., Toxicol. Appl. Pharmacol., 18, 35-40 (1971)]. For example, a PEG adduct of adenosine deaminase was approved in the United States for use in humans for the treatment of severe combined immunodeficiency syndrome. A second advantage afforded by the conjugation of PEG is that of effectively reducing the immunogenicity and antigenicity of heterologous proteins. For example, a PEG adduct of a human protein might be useful for the treatment of

disease in other mammalian species without the risk of triggering a severe immune response.

Polymers such as PEG may be conveniently attached to one or more reactive amino acid residues in a protein such as the alpha-amino group of the amino-terminal amino acid, the epsilon amino groups of lysine side chains, the sulfhydryl groups of cysteine side chains, the carboxyl groups of aspartyl and glutamyl side chains, the alpha-carboxyl group of the carboxyl-terminal amino acid, tyrosine side chains, or to activated derivatives of glycosyl chains attached to certain asparagine, serine or threonine residues.

Numerous activated forms of PEG suitable for direct reaction with proteins have been described. Useful PEG reagents for reaction with protein amino groups include active esters of carboxylic acid or carbonate derivatives, particularly those in which the leaving groups are N-hydroxysuccinimide, p-nitrophenol, imidazole or 1-hydroxy-2-nitrobenzene-4-sulfonate. PEG derivatives containing maleimido or haloacetyl groups are useful reagents for the modification of protein free sulfhydryl groups. Likewise, PEG reagents containing amino, hydrazine or hydrazide groups are useful for reaction with aldehydes generated by periodate oxidation of carbohydrate groups in proteins.

It is an object of the present invention to provide a factor causing growth of early hematopoietic progenitor cells.

Summary of the Invention

According to the present invention, novel factors, referred to herein as "stem cell factors" (SCF) having the ability to stimulate growth of primitive progenitors including early hematopoietic progenitor cells are provided. These SCFs also are able to

stimulate non-hematopoietic stem cells such as neural stem cells and primordial germ stem cells. Such factors include purified naturally-occurring stem cell factors. The invention also relates to non-naturally-occurring polypeptides having amino acid sequences sufficiently duplicative of that of naturally-occurring stem cell factor to allow possession of a hematopoietic biological activity of naturally occurring stem cell factor.

The present invention also provides isolated DNA sequences for use in securing expression in procaryotic or eukaryotic host cells of polypeptide products having amino acid sequences sufficiently duplicative of that of naturally-occurring stem cell factor to allow possession of a hematopoietic biological activity of naturally occurring stem cell factor. Such DNA sequences include:

(a) DNA sequences set out in Figures 14B, 14C, 15B, 15C, 42 and 44 or their complementary strands;

(b) DNA sequences which hybridize to the DNA sequences defined in (a) or fragments thereof; and

(c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) and (b).

Also provided are vectors containing such DNA sequences, and host cells transformed or transfected with such vectors. Also comprehended by the invention are methods of producing SCF by recombinant techniques, and methods of treating disorders. Additionally, pharmaceutical compositions including SCF and antibodies specifically binding SCF are provided.

The invention also relates to a process for the efficient recovery of stem cell factor from a material containing SCF, the process comprising the steps of ion exchange chromatographic separation and/or reverse phase liquid chromatographic separation.

The present invention also provides a biologically-active adduct having prolonged *in vivo* half-life and enhanced potency in mammals, comprising SCF covalently conjugated to a water-soluble polymer such as polyethylene glycol or copolymers of polyethylene glycol and polypropylene glycol, wherein said polymer is unsubstituted or substituted at one end with an alkyl group. Another aspect of this invention resides in a process for preparing the adduct described above, comprising reacting the SCF with a water-soluble polymer having at least one terminal reactive group and purifying the resulting adduct to produce a product with extended circulating half-life and enhanced biological activity.

Brief Description of the Drawings

Figure 1 is an anion exchange chromatogram from the purification of mammalian SCF.

Figure 2 is a gel filtration chromatogram from the purification of mammalian SCF.

Figure 3 is a wheat germ agglutinin-agarose chromatogram from the purification of mammalian SCF.

Figure 4 is a cation exchange chromatogram from the purification of mammalian SCF.

Figure 5 is a C_4 chromatogram from the purification of mammalian SCF.

Figure 6 shows sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (SDS-PAGE) of C_4 column fractions from Figure 5.

Figure 7 is an analytical C_4 chromatogram of mammalian SCF.

Figure 8 shows SDS-PAGE of C_4 column fractions from Figure 7.

Figure 9 shows SDS-PAGE of purified mammalian SCF and deglycosylated mammalian SCF.

Figure 10 is an analytical C_4 chromatogram of purified mammalian SCF.

Figure 11 shows the amino acid sequence of mammalian SCF derived from protein sequencing.

Figure 12 shows

A. oligonucleotides for rat SCF cDNA
B. oligonucleotides for human SCF DNA
C. universal oligonucleotides.

Figure 13 shows

A. a scheme for polymerase chain reaction (PCR) amplification of rat SCF cDNA
B. a scheme for PCR amplification of human SCF cDNA.

Figure 14 shows

A. sequencing strategy for rat genomic DNA
B. the nucleic acid sequence of rat genomic DNA.
C. the nucleic acid sequence of rat SCF cDNA and amino acid sequence of rat SCF protein.

Figure 15 shows

A. the strategy for sequencing human genomic DNA

B. the nucleic acid sequence of human genomic DNA

C. the composite nucleic acid sequence of human SCF cDNA and amino acid sequence of SCF protein.

Figure 16 shows the aligned amino acid sequences of human, monkey, dog, mouse, and rat SCF protein.

Figure 17 shows the structure of mammalian cell expression vector V19.8 SCF.

Figure 18 shows the structure of mammalian CHO cell expression vector pDSVE.1.

Figure 19 shows the structure of E. coli expression vector pCFM1156.

Figure 20 shows

A. a radioimmunoassay of mammalian SCF

B. SDS-PAGE of immune-precipitated mammalian SCF.

Figure 21 shows Western analysis of recombinant human SCF.

Figure 22 shows Western analysis of recombinant rat SCF.

Figure 23 is a bar graph showing the effect of COS-1 cell-produced recombinant rat SCF on bone marrow transplantation.

Figure 24 shows the effect of recombinant rat SCF on curing the macrocytic anemia of Steel mice.

Figure 25 shows the peripheral white blood cell count (WBC) of Steel mice treated with recombinant rat SCF.

Figure 26 shows the platelet counts of Steel mice treated with recombinant rat SCF.

Figure 27 shows the differential WBC count for Steel mice treated with recombinant rat SCF^{1-164} PEG25.

Figure 28 shows the lymphocyte subsets for Steel mice treated with recombinant rat SCF^{1-164} PEG25.

Figure 29 shows the effect of recombinant human sequence SCF treatment of normal primates in increasing peripheral WBC count.

Figure 30 shows the effect of recombinant human sequence SCF treatment of normal primates in increasing hematocrits and platelet numbers.

Figure 31 shows photographs of

A. human bone marrow colonies stimulated by recombinant human SCF^{1-162}

B. Wright-Giemsa stained cells from colonies in Figure 31 A.

Figure 32 shows SDS-PAGE of S-Sepharose column fractions from chromatogram shown in Figure 33

A. with reducing agent

B. without reducing agent.

Figure 33 is a chromatogram of an S-Sepharose column of E. coli derived recombinant human SCF.

Figure 34 shows SDS-PAGE of C_4 column fractions from chromatogram showing Figure 35

A. with reducing agent

B. without reducing agent.

Figure 35 is a chromatogram of a C_4 column of E. coli derived recombinant human SCF.

Figure 36 is a chromatogram of a Q-Sepharose column of CHO derived recombinant rat SCF.

Figure 37 is a chromatogram of a C_4 column of CHO derived recombinant rat SCF.

Figure 38 shows SDS-PAGE of C_4 column fractions from chromatogram shown in Figure 37.

Figure 39 shows SDS-PAGE of purified CHO derived recombinant rat SCF before and after de-glycosylation.

Figure 40 shows

A. gel filtration chromatography of recombinant rat pegylated SCF^{1-164} reaction mixture

B. gel filtration chromatography of recombinant rat SCF^{1-164}, unmodified.

Figure 41 shows labelled SCF binding to fresh leukemic blasts.

Figure 42 shows human SCF cDNA sequence obtained from the HT1080 fibrosarcoma cell line.

Figure 43 shows an autoradiograph from COS-7 cells expressing human SCF^{1-248} and CHO cells expressing human SCF^{1-164}.

Figure 44 shows human SCF cDNA sequence obtained from the 5637 bladder carcinoma cell line.

Figure 45 shows the enhanced survival of irradiated mice after SCF treatment.

Figure 46 shows the enhanced survival of irradiated mice after bone marrow transplantation with 5% of a femur and SCF treatment.

Figure 47 shows the enhanced survival of irradiated mice after bone marrow transplantation with 0.1 and 20% of a femur and SCF treatment.

Numerous aspects and advantages of the invention will be apparent to those skilled in the art upon consideration of the following detailed description which provides illustrations of the practice of the invention in its presently-preferred embodiments.

Detailed Description of the Invention

According to the present invention, novel stem cell factors and DNA sequences coding for all or part of such SCFs are provided. The term "stem cell factor" or "SCF" as used herein refers to naturally-occurring SCF (e.g. natural human SCF) as well as non-naturally occurring (i.e., different from naturally occurring) polypeptides having amino acid sequences and glycosylation sufficiently duplicative of that of naturally-occurring stem cell factor to allow possession of a hematopoietic biological activity of naturally-

occurring stem cell factor. Stem cell factor has the ability to stimulate growth of early hematopoietic progenitors which are capable of maturing to erythroid, megakaryocyte, granulocyte, lymphocyte, and macrophage cells. SCF treatment of mammals results in absolute increases in hematopoietic cells of both myeloid and lymphoid lineages. One of the hallmark characteristics of stem cells is their ability to differentiate into both myeloid and lymphoid cells [Weissman, Science, 241, 58-62 (1988)]. Treatment of Steel mice (Example 8B) with recombinant rat SCF results in increases of granulocytes, monocytes, erythrocytes, lymphocytes, and platelets. Treatment of normal primates with recombinant human SCF results in increases in myeloid and lymphoid cells (Example 8C).

There is embryonic expression of SCF by cells in the migratory pathway and homing sites of melanoblasts, germ cells, hematopoietic cells, brain and spinal chord.

Early hematopoietic progenitor cells are enriched in bone marrow from mammals which has been treated with 5-Fluorouracil (5-FU). The chemotherapeutic drug 5-FU selectively depletes late hematopoietic progenitors. SCF is active on post 5-FU bone marrow.

The biological activity and pattern of tissue distribution of SCF demonstrates its central role in embryogenesis and hematopoiesis as well as its capacity for treatment of various stem cell deficiencies.

The present invention provides DNA sequences which include: the incorporation of codons "preferred" for expression by selected nonmammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences which facilitate construction of readily-expressed vectors.

The present invention also provides DNA sequences coding for polypeptide analogs or derivatives of SCF which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (i.e., deletion analogs containing less than all of the residues specified for SCF; substitution analogs, wherein one or more residues specified are replaced by other residues; and addition analogs wherein one or more amino acid residues is added to a terminal or medial portion of the polypeptide) and which share some or all the properties of naturally-occurring forms. The present invention specifically provides DNA sequences encoding the full length unprocessed amino acid sequence as well as DNA sequences encoding the processed form of SCF.

Novel DNA sequences of the invention include sequences useful in securing expression in procaryotic or eucaryotic host cells of polypeptide products having at least a part of the primary structural conformation and one or more of the biological properties of naturally-occurring SCF. DNA sequences of the invention specifically comprise: (a) DNA sequences set forth in Figures 14B, 14C, 15B, 15C, 42 and 44 or their complementary strands; (b) DNA sequences which hybridize (under hybridization conditions disclosed in Example 3 or more stringent conditions) to the DNA sequences in Figures 14B, 14C, 15B, 15C, 42, and 44 or to fragments thereof; and (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences in Figures 14B, 14C, 15B, 15C, 42, and 44. Specifically comprehended in parts (b) and (c) are genomic DNA sequences encoding allelic variant forms of SCF and/or encoding SCF from other mammalian species, and manufactured DNA sequences encoding SCF, fragments of SCF, and analogs of SCF. The DNA sequences may incorporate codons facilitating transcription and translation of messenger RNA in microbial hosts. Such

manufactured sequences may readily be constructed according to the methods of Alton et al., PCT published application WO 83/04053.

According to another aspect of the present invention, the DNA sequences described herein which encode polypeptides having SCF activity are valuable for the information which they provide concerning the amino acid sequence of the mammalian protein which have heretofore been unavailable. The DNA sequences are also valuable as products useful in effecting the large scale synthesis of SCF by a variety of recombinant techniques. Put another way, DNA sequences provided by the invention are useful in generating new and useful viral and circular plasmid DNA vectors, new and useful transformed and transfected procaryotic and eucaryotic host cells (including bacterial and yeast cells and mammalian cells grown in culture), and new and useful methods for cultured growth of such host cells capable of expression of SCF and its related products.

DNA sequences of the invention are also suitable materials for use as labeled probes in isolating human genomic DNA encoding SCF and other genes for related proteins as well as cDNA and genomic DNA sequences of other mammalian species. DNA sequences may also be useful in various alternative methods of protein synthesis (e.g., in insect cells) or in genetic therapy in humans and other mammals. DNA sequences of the invention are expected to be useful in developing transgenic mammalian species which may serve as eucaryotic "hosts" for production of SCF and SCF products in quantity. See, generally, Palmiter et al., Science 222, 809-814 (1983).

The present invention provides purified and isolated naturally-occurring SCF (i.e. purified from nature or manufactured such that the primary, secondary and tertiary conformation, and the glycosylation pattern

are identical to naturally-occurring material) as well as non-naturally occurring polypeptides having a primary structural conformation (i.e., continuous sequence of amino acid residues) and glycosylation sufficiently duplicative of that of naturally occurring stem cell factor to allow possession of a hematopoietic biological activity of naturally occurring SCF. Such polypetides include derivatives and analogs.

In a preferred embodiment, SCF is characterized by being the product of procaryotic or eucaryotic host expression (e.g., by bacterial, yeast, higher plant, insect and mammalian cells in culture) of exogenous DNA sequences obtained by genomic or cDNA cloning or by gene synthesis. That is, in a preferred embodiment, SCF is "recombinant SCF." The products of expression in typical yeast (e.g., Saccharomyces cerevisiae) or procaryote (e.g., E. coli) host cells are free of association with any mammalian proteins. The products of expression in vertebrate [e.g., non-human mammalian (e.g. COS or CHO) and avian] cells are free of association with any human proteins. Depending upon the host employed, polypeptides of the invention may be glycosylated with mammalian or other eucaryotic carbohydrates or may be non-glycosylated. The host cell can be altered using techniques such as those described in Lee et al. J. Biol. Chem. 264, 13848 (1989) hereby incorporated by reference. Polypeptides of the invention may also include an initial methionine amino acid residue (at position -1).

In addition to naturally-occurring allelic forms of SCF, the present invention also embraces other SCF products such as polypeptide analogs of SCF. Such analogs include fragments of SCF. Following the procedures of the above-noted published application by Alton et al. (WO 83/04053), one can readily design and manufacture genes coding for microbial expression of

polypeptides having primary conformations which differ from that herein specified for in terms of the identity or location of one or more residues (e.g., substitutions, terminal and intermediate additions and deletions). Alternately, modifications of cDNA and genomic genes can be readily accomplished by well-known site-directed mutagenesis techniques and employed to generate analogs and derivatives of SCF. Such products share at least one of the biological properties of SCF but may differ in others. As examples, products of the invention include those which are foreshortened by e.g., deletions; or those which are more stable to hydrolysis (and, therefore, may have more pronounced or longer-lasting effects than naturally-occurring); or which have been altered to delete or to add one or more potential sites for O-glycosylation and/or N-glycosylation or which have one or more cysteine residues deleted or replaced by, e.g., alanine or serine residues and are potentially more easily isolated in active form from microbial systems; or which have one or more tyrosine residues replaced by phenylalanine and bind more or less readily to target proteins or to receptors on target cells. Also comprehended are polypeptide fragments duplicating only a part of the continuous amino acid sequence or secondary conformations within SCF, which fragments may possess one property of SCF (e.g., receptor binding) and not others (e.g., early hematopoietic cell growth activity). It is noteworthy that activity is not necessary for any one or more of the products of the invention to have therapeutic utility [see, Weiland et al., Blut, 44, 173-175 (1982)] or utility in other contexts, such as in assays of SCF antagonism. Competitive antagonists may be quite useful in, for example, cases of overproduction of SCF or cases of human leukemias where the malignant cells overexpress receptors for SCF, as indicated by the overexpression of SCF receptors in leukemic blasts (Example 13).

Of applicability to polypeptide analogs of the invention are reports of the immunological property of synthetic peptides which substantially duplicate the amino acid sequence extant in naturally-occurring proteins, glycoproteins and nucleoproteins. More specifically, relatively low molecular weight polypeptides have been shown to participate in immune reactions which are similar in duration and extent to the immune reactions of physiologically-significant proteins such as viral antigens, polypeptide hormones, and the like. Included among the immune reactions of such polypeptides is the provocation of the formation of specific antibodies in immunologically-active animals [Lerner et al., Cell, 23, 309-310 (1981); Ross et al., Nature, 294, 654-656 (1981); Walter et al., Proc. Natl. Acad. Sci. USA, 77, 5197-5200 (1980); Lerner et al., Proc. Natl. Acad. Sci. USA, 78, 3403-3407 (1981); Walter et al., Proc. Natl. Acad. Sci. USA, 78, 4882-4886 (1981); Wong et al., Proc. Natl. Acad. Sci. USA, 79, 5322-5326 (1982); Baron et al., Cell, 28, 395-404 (1982); Dressman et al., Nature, 295, 185-160 (1982); and Lerner, Scientific American, 248, 66-74 (1983)]. See, also, Kaiser et al. [Science, 223, 249-255 (1984)] relating to biological and immunological properties of synthetic peptides which approximately share secondary structures of peptide hormones but may not share their primary structural conformation.

The present invention also includes that class of polypeptides coded for by portions of the DNA complementary to the protein-coding strand of the human cDNA or genomic DNA sequences of SCF, i.e., "complementary inverted proteins" as described by Tramontano et al. [Nucleic Acid Res., 12, 5049-5059 (1984)].

Representative SCF polypeptides of the present invention include but are not limited to SCF^{1-148},

SCF^{1-162}, SCF^{1-164}, SCF^{1-165} and SCF^{1-183} in Figure 15C; SCF^{1-185}, SCF^{1-188}, SCF^{1-189} and SCF^{1-248} in Figure 42; and SCF^{1-157}, SCF^{1-160}, SCF^{1-161} and SCF^{1-220} in Figure 44.

SCF can be purified using techniques known to those skilled in the art. The subject invention comprises a method of purifying SCF from an SCF containing material such as conditioned media or human urine, serum, the method comprising one or more of steps such as the following: subjecting the SCF containing material to ion exchange chromatography (either cation or anion exchange chromatography); subjecting the SCF containing material to reverse phase liquid chromatographic separation involving, for example, an immobilized C_4 or C_6 resin; subjecting the fluid to immobilized-lectin chromatography, i.e., binding of SCF to the immobilized lectin, and eluting with the use of a sugar that competes for this binding. Details in the use of these methods will be apparent from the descriptions given in Examples 1, 10, and 11 for the purification of SCF. The techniques described in Example 2 of the Lai et al. U.S. patent 4,667,016, hereby incorporated by reference are also useful in purifying stem cell factor.

Isoforms of SCF are isolated using standard techniques such as the techniques set forth in commonly owned U.S. Ser. No. 421,444 entitled Erythropoietin Isoforms, filed October 13, 1989, hereby incorporated by reference.

Also comprehended by the invention are pharmaceutical compositions comprising therapeutically effective amounts of polypeptide products of the invention together with suitable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers useful in SCF therapy. A "therapeutically effective amount" as used herein refers

to that amount which provides a therapeutic effect for a given condition and administration regimen. Such compositions are liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl., acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent adsorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), solubilizing agents (e.g., glycerol, polyethylene glycol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the protein (described in Example 12 below), complexation with metal ions, or incorporation of the material into or onto particulate preparations of polymeric compounds such as polylactic acid, polglycolic acid, hydrogels, etc. or into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance of SCF. The choice of composition will depend on the physical and chemical properties of the protein having SCF activity. For example, a product derived from a membrane-bound form of SCF may require a formulation containing detergent. Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines) and SCF coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate

forms, protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

The invention also comprises compositions including one or more additional hematopoietic factors such as EPO, G-CSF, GM-CSF, CSF-1, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IGF-I, or LIF (Leukemic Inhibitory Factor).

Polypeptides of the invention may be "labeled" by association with a detectable marker substance (e.g., radiolabeled with ^{125}I or biotinylated) to provide reagents useful in detection and quantification of SCF or its receptor bearing cells in solid tissue and fluid samples such as blood or urine.

Biotinylated SCF is useful in conjunction with immobilized streptavidin to purge leukemic blasts from bone marrow in autologous bone marrow transplantation. Biotinylated SCF is useful in conjunction with immobilized streptavidin to enrich for stem cells in autologous or allogeneic stem cells in autologous or allogeneic bone marrow transplantation. Toxin conjugates of SCF, such as ricin [Uhr, Prog. Clin. Biol. Res. 288, 403-412 (1989)] diptheria toxin [Moolten, J. Natl. Con. Inst., 55, 473-477 (1975)], and radioisotopes are useful for direct anti-neoplastic therapy (Example 13) or as a conditioning regimen for bone marow transplantation.

Nucleic acid products of the invention are useful when labeled with detectable markers (such as radiolabels and non-isotopic labels such as biotin) and employed in hybridization processes to locate the human SCF gene position and/or the position of any related gene family in a chromosomal map. They are also useful for identifying human SCF gene disorders at the DNA level and used as gene markers for identifying

neighboring genes and their disorders. The human SCF gene is encoded on chromosome 12, and the murine SCF gene maps to chromosome 10 at the *Sl* locus.

SCF is useful, alone or in combination with other therapy, in the treatment of a number of hematopoietic disorders. SCF can be used alone or with one or more additional hematopoietic factors such as EPO, G-CSF, GM-CSF, CSF-1, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-1, IGF-I or LIF in the treatment of hematopoietic disorders.

There is a group of stem cell disorders which are characterized by a reduction in functional marrow mass due to toxic, radiant, or immunologic injury and which may be treatable with SCF. Aplastic anemia is a stem cell disorder in which there is a fatty replacement of hematopoietic tissue and pancytopenia. SCF enhances hematopoietic proliferation and is useful in treating aplastic anemia (Example 8B). Steel mice are used as a model of human aplastic anemia [Jones, Exp. Hematol., 11, 571-580 (1983)]. Promising results have been obtained with the use of a related cytokine, GM-CSF in the treatment of aplastic anemia [Antin, et al., Blood, 70, 129a (1987)]. Paroxysmal nocturnal hemoglobinuria (PNH) is a stem cell disorder characterized by formation of defective platelets and granulocytes as well as abnormal erythrocytes.

There are many diseases which are treatable with SCF. These include the following: myelofibrosis, myelosclerosis, osteopetrosis, metastatic carcinoma, acute leukemia, multiple myeloma, Hodgkin's disease, lymphoma, Gaucher's disease, Niemann-Pick disease, Letterer-Siwe disease, refractory erythroblastic anemia, Di Guglielmo syndrome, congestive splenomegaly, Hodgkin's disease, Kala azar, sarcoidosis, primary splenic pancytopenia, miliary tuberculosis, disseminated fungus disease, Fulminating septicemia, malaria, vitamin

B_{12} and folic acid deficiency, pyridoxine deficiency, Diamond Blackfan anemia, hypopigmentation disorders such as piebaldism and vitiligo. The erythroid, megakaryocyte, and granulocytic stimulatory properties of SCF are illustrated in Example 8B and 8C.

Enhancement of growth in non-hematopoietic stem cells such as primordial germ cells, neural crest derived melanocytes, commissural axons originating from the dorsal spinal cord, crypt cells of the gut, mesonephric and metanephric kidney tubules, and olfactory bulbs is of benefit in states where specific tissue damage has occurred to these sites. SCF is useful for treating neurological damage and is a growth factor for nerve cells. SCF is useful during in vitro fertilization procedures or in treatment of infertility states. SCF is useful for treating intestinal damage resulting from irradiation or chemotherapy.

There are stem cell myeloproliferative disorders such as polycythemia vera, chronic myelogenous leukemia, myeloid mataplasia, primary thrombocythemia, and acute leukemias which are treatable with SCF, anti-SCF antibodies, or SCF-toxin conjugates.

There are numerous cases which document the increased proliferation of leukemic cells to the hematopoietic cell growth factors G-CSF, GM-CSF, and IL-3 [Delwel, et al., Blood, 72, 1944-1949 (1988)]. Since the success of many chemotherapeutic drugs depends on the fact that neoplastic cells cycle more actively than normal cells, SCF alone or in combination with other factors acts as a growth factor for neoplastic cells and sensitizes them to the toxic effects of chemotherapeutic drugs. The overexpression of SCF receptors on leukemic blasts is shown in Example 13.

A number of recombinant hematopoietic factors are undergoing investigation for their ability to shorten the leukocyte nadir resulting from chemotherapy

and radiation regimens. Although these factors are very useful in this setting, there is an early hematopoietic compartment which is damaged, especially by radiation, and has to be repopulated before these later-acting growth factors can exert their optimal action. The use of SCF alone or in combination with these factors further shortens or eliminates the leukocyte and platelet nadir resulting from chemotherapy or radiation treatment. In addition, SCF allows for a dose intensification of the anti-neoplastic or irradiation regimen (Example 19).

SCF is useful for expanding early hematopoietic progenitors in syngeneic, allogeneic, or autologous bone marrow transplantation. The use of hematopoietic growth factors has been shown to decrease the time for neutrophil recovery after transplantation [Donahue, et al., Nature, 321, 872-875 (1986) and Welte et al., J. Exp. Med., 165, 941-948, (1987)]. For bone marrow transplantation, the following three scenarios are used alone or in combination: a donor is treated with SCF alone or in combination with other hematopoietic factors prior to bone marrow aspiration or peripheral blood leucophoresis to increase the number of cells available for transplantation; the bone marrow is treated in vitro to activate or expand the cell number prior to transplantation; finally, the recipient is treated to enhance engraftment of the donor marrow.

SCF is useful for enhancing the efficiency of gene therapy based on transfecting (or infecting with a retroviral vector) hematopoietic stem cells. SCF permits culturing and multiplication of the early hematopoietic progenitor cells which are to be transfected. The culture can be done with SCF alone or in combination with IL-6, IL-3, or both. Once tranfected, these cells are then infused in a bone marrow transplant into patients suffering from genetic

disorders. [Lim, Proc. Natl. Acad. Sci, 86, 8892-8896 (1989)]. Examples of genes which are useful in treating genetic disorders include adenosine deaminase, glucocerebrosidase, hemoglobin, and cystic fibrosis.

SCF is useful for treatment of acquired immune deficiency (AIDS) or severe combined immunodeficiency states (SCID) alone or in combination with other factors such as IL-7 (see Example 14). Illustrative of this effect is the ability of SCF therapy to increase the absolute level of circulating T-helper (CD4+, OKT_4+) lymphocytes. These cells are the primary cellular target of human immunodeficiency virus (HIV) leading to the immunodeficiency state in AIDS patients [Montagnier, in Human T-Cell Leukemia/Lymphoma Virus, ed. R.C. Gallo, Cold Spring Harbor, New York, 369-379 (1984)]. In addition, SCF is useful for combatting the myelosuppressive effects of anti-HIV drugs such as AZT [Gogu Life Sciences, 45, No. 4 (1989)].

SCF is useful for enhancing hematopoietic recovery after acute blood loss.

In vivo treatment with SCF is useful as a boost to the immune system for fighting neoplasia (cancer). An example of the therapeutic utility of direct immune function enhancement by a recently cloned cytokine (IL-2) is described in Rosenberg et al., N. Eng. J. Med., 313 1485 (1987).

The administration of SCF with other agents such as one or more other hematopoietic factors, is temporally spaced or given together. Prior treatment with SCF enlarges a progenitor population which responds to terminally-acting hematopoietic factors such as G-CSF or EPO. The route of administration may be intravenous, intraperitoneal sub-cutaneous, or intramuscular.

The subject invention also relates to antibodies specifically binding stem cell factor.

Example 7 below describes the production of polyclonal antibodies. A further embodiment of the invention is monoclonal antibodies specifically binding SCF (see Example 20). In contrast to conventional antibody (polyclonal) preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. Monoclonal antibodies are useful to improve the selectivity and specificity of diagnostic and analytical assay methods using antigen-antibody binding. Also, they are used to neutralize or remove SCF from serum. A second advantage of monoclonal antibodies is that they can be synthesized by hybridoma cells in culture, uncontaminated by other immunoglobulins. Monoclonal antibodies may be prepared from supernatants of cultured hybridoma cells or from ascites induced by intraperitoneal inoculation of hybridoma cells into mice. The hybridoma technique described originally by Köhler and Milstein [Eur. J. Immunol. 6, 511-519 (1976)] has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

The following examples are offered to more fully illustrate the invention, but are not to be construed as limiting the scope thereof.

EXAMPLE 1

Purification/Characterization of Stem Cell Factor from Buffalo Rat Liver Cell Conditoned Medium

A. In Vitro Biological Assays

1. HPP-CFC Assay

There are a variety of biological activities which can be attributed to the natural mammalian rat SCF

as well as the recombinant rat SCF protein. One such activity is its effect on early hematopoietic cells. This activity can be measured in a High Proliferative Potential Colony Forming Cell (HPP-CFC) assay [Zsebo, et al., supra (1988)]. To investigate the effects of factors on early hematopoietic cells, the HPP-CFC assay system utilizes mouse bone marrow derived from animals 2 days after 5-fluorouracil (5-FU) treatment. The chemotherapeutic drug 5-FU selectively depletes late hematopoietic progenitors, allowing for detection of early progenitor cells and hence factors which act on such cells. The rat SCF is plated in the presence of CSF-1 or IL-6 in semi-solid agar cultures. The agar cultures contain McCoys complete medium (GIBCO), 20% fetal bovine serum, 0.3% agar, and $2x10^5$ bone marrow cells/ml. The McCoys complete medium contains the following components: 1xMcCoys medium supplemented with 0.1 mM pyruvate, 0.24x essential amino acids, 0.24x non-essential amino acids, 0.027% sodium bicarbonate, 0.24x vitamins, 0.72 mM glutamine, 25 µg/ml L-serine, and 12 µg/ml L-asparagine. The bone marrow cells are obtained from Balb/c mice injected i.v. with 150 mg/kg 5-FU. The femurs are harvested 2 days post 5-FU treatment of the mice and bone marrow is flushed out. The red blood cells are lysed with red blood cell lysing reagent (Becton Dickenson) prior to plating. Test substances are plated with the above mixture in 30 mm dishes. Fourteen days later the colonies (>1 mm in diameter) which contain thousands of cells are scored. This assay was used throughout the purification of natural mammalian cell-derived rat SCF.

In a typical assay, rat SCF causes the proliferation of approximately 50 HPP-CFC per 200,000 cells plated. The rat SCF has a synergistic activity on 5-FU treated mouse bone marrow cells; HPP-CFC colonies will not form in the presence of single factors but the

combination of SCF and CSF-1 or SCF and IL-6 is active in this assay.

2. MC/9 Assay

Another useful biological activity of both naturally-derived and recombinant rat SCF is the ability to cause the proliferation of the IL-4 dependent murine mast cell line, MC/9 (ATCC CRL 8306). MC/9 cells are cultured with a source of IL-4 according to the ATCC CRL 8306 protocol. The medium used in the bioassay is RPMI 1640, 4% fetal bovine serum, $5x10^{-5}$M 2-mercaptoethanol, and 1x glutamine-pen-strep. The MC/9 cells proliferate in response to SCF without the requirement for other growth factors. This proliferation is measured by first culturing the cells for 24 h without growth factors, plating 5000 cells in each well of 96 well plates with test sample for 48h, pulsing for 4 h with 0.5 uCi ^{3}H-thymidine (specific activity 20 Ci/mmol), harvesting the solution onto glass fiber filters, and then measuring specifically-bound radioactivity. This assay was used in the purification of mammalian cell derived rat SCF after the ACA 54 gel filtration step, section C2 of this Example. Typically, SCF caused a 4-10 fold increase in CPM over background.

3. CFU-GM

The action of purified mammalian SCF, both naturally-derived and recombinant, free from interfering colony stimulating factors (CSFs), on normal undepleted mouse bone marrow has been ascertained. A CFU-GM assay [Broxmeyer et al. Exp. Hematol., 5, 87 (1977)] is used to evaluate the effect of SCF on normal marrow. Briefly, total bone marrow cells after lysis of red blood cells are plated in semi-solid agar cultures containing the test substance. After 10 days, the colonies containing clusters of >40 cells are scored.

The agar cultures can be dried down onto glass slides and the morphology of the cells can be determined via specific histological stains.

On normal mouse bone marrow, the purified mammalian rat SCF was a pluripotential CSF, stimulating the growth of colonies consisting of immature cells, neutrophils, macrophages, eosinophils, and megakaryocytes without the requirement for other factors. From 200,000 cells plated, over 100 such colonies grow over a 10 day period. Both rat and human recombinant SCF stimulate the production of erythroid cells in combination with EPO, see Example 9.

B. Conditioned Medium

Buffalo rat liver (BRL) 3A cells, from the American Type Culture Collection (ATCC CRL 1442), were grown on microcarriers in a 20 liter perfusion culture system for the production of SCF. This system utilizes a Biolafitte fermenter (Model ICC-20) except for the screens used for retention of microcarriers and the oxygenation tubing. The 75 micron mesh screens are kept free of microcarrier clogging by periodic back flushing achieved through a system of check valves and computer-controlled pumps. Each screen alternately acts as medium feed and harvest screen. This oscillating flow pattern ensures that the screens do not clog. Oxygenation was provided through a coil of silicone tubing (50 feet long, 0.25 inch ID, 0.03 inch wall). The growth medium used for the culture of BRL 3A cells was Minimal Essential Medium (with Earle's Salts) (GIBCO), 2 mM glutamine, 3 g/L glucose, tryptose phosphate (2.95 g/L), 5% fetal bovine serum and 5% fetal calf serum. The harvest medium was identical except for the omission of serum. The reactor contained Cytodex 2 microcarriers (Pharmacia) at a concentration of 5 g/L and was seeded

with 3 x 10^9 BRL 3A cells grown in roller bottles and removed by trypsinization. The cells were allowed to attach to and grow on the microcarriers for eight days. Growth medium was perfused through the reactor as needed based on glucose consumption. The glucose concentration was maintained at approximately 1.5 g/L. After eight days, the reactor was perfused with six volumes of serum free medium to remove most of the serum (protein concentration < 50 ug/ml). The reactor was then operated batchwise until the glucose concentration fell below 2 g/L. From this point onward, the reactor was operated at a continuous perfusion rate of approximately 10 L/day. The pH of the culture was maintained at 6.9 ± 0.3 by adjusting the CO_2 flow rate. The dissolved oxygen was maintained higher than 20% of air saturation by supplementing with pure oxygen as necessary. The temperature was maintained at 37 ± 0.5°C.

Approximately 336 liters of serum free conditioned medium was generated from the above system and was used as the starting material for the purification of natural mammalian cell-derived rat SCF.

C. Purification

All purification work was carried out at 4°C unless indicated otherwise.

1. DEAE-cellulose Anion Exchange Chromatography

Conditioned medium generated by serum-free growth of BRL 3A cells was clarified by filtration through 0.45 μ Sartocapsules (Sartorius). Several different batches (41 L, 27 L, 39 L, 30.2 L, 37.5 L, and 161 L) were separately subjected to concentration, diafiltration/buffer exchange, and DEAE-cellulose anion exchange chromatography, in similar fashion for each batch. The DEAE-cellulose pools were then combined and

processed further as one batch in sections C2-5 of this Example. To illustrate, the handling of the 41 L batch was as follows. The filtered conditioned medium was concentrated to ~700 ml using a Millipore Pellicon tangential flow ultrafiltration apparatus with four 10,000 molecular weight cutoff polysulfone membrane cassettes (20 ft^2 total membrane area; pump rate ~1095 ml/min and filtration rate 250-315 ml/min). Diafiltration/buffer exchange in preparation for anion exchange chromatography was then accomplished by adding 500 ml of 50 mM Tris-HCl, pH 7.8 to the concentrate, reconcentrating to 500 ml using the tangential flow ultrafiltration apparatus, and repeating this six additional times. The concentrated/diafiltered preparation was finally recovered in a volume of 700 ml. The preparation was applied to a DEAE-cellulose anion exchange column (5 x 20.4 cm; Whatman DE-52 resin) which had been equilibrated with the 50 mM Tris-HCl, pH 7.8 buffer. After sample application, the column was washed with 2050 ml of the Tris-HCl buffer, and a salt gradient (0-300 mM NaCl in the Tris-HCl buffer; 4 L total volume) was applied. Fractions of 15 ml were collected at a flow rate of 167 ml/h. The chromatography is shown in Figure 1. HPP-CFC colony number refers to biological activity in the HPP-CFC assay; 100 µl from the indicated fractions was assayed. Fractions collected during the sample application and wash are not shown in the Figure; no biological activity was detected in these fractions.

The behavior of all conditioned media batches subjected to the concentration, diafiltration/buffer exchange, and anion exchange chromatography was similar. Protein concentrations for the batches, determined by the method of Bradford [Anal. Biochem. 72, 248-254 (1976)] with bovine serum albumin as standard were in the range 30-50 µg/ml. The total volume of conditioned medium utilized for this preparation was about 336 L.

2. ACA 54 Gel Filtration Chromatography

Fractions having biological activity from the DEAE-cellulose columns run for each of the six conditioned media batches referred to above (for example, fractions 87-114 for the run shown in Figure 1) were combined (total volume 2900 ml) and concentrated to a final volume of 74 ml with the use of Amicon stirred cells and YM10 membranes. This material was applied to an ACA 54 (LKB) gel filtration column (Figure 2) equilibrated in 50 mM Tris-HCl, 50 mM NaCl, pH 7.4. Fractions of 14 ml were collected at a flow rate of 70 ml/h. Due to inhibitory factors co-eluting with the active fractions, the peak of activity (HPP-CFC colony number) appears split; however, based on previous chromatograms, the activity co-elutes with the major protein peak and therefore one pool of the fractions was made.

3. Wheat Germ Agglutinin-Agarose Chromatography

Fractions 70-112 from the ACA 54 gel filtration column were pooled (500 ml). The pool was divided in half and each half subjected to chromatography using a wheat germ agglutinin-agarose column (5 x 24.5 cm; resin from E-Y Laboratories, San Mateo, CA; wheat germ agglutinin recognizes certain carbohydrate structures) equilibrated in 20 mM Tris-HCl, 500 mM NaCl, pH 7.4. After the sample applications, the column was washed with about 2200 ml of the column buffer, and elution of bound material was then accomplished by applying a solution of 350 mM N-acetyl-D-glucosamine dissolved in the column buffer, beginning at fraction ~210 in Figure 3. Fractions of 13.25 ml were collected at a flow rate of 122 ml/h. One of the chromatographic runs is shown in Figure 3. Portions of the fractions to be assayed were dialyzed against phosphate-buffered saline; 5 ul of the dialyzed

materials were placed into the MC/9 assay (cpm values in Figure 3) and 10 µl into the HPP-CFC assay (colony number values in Figure 3). It can be seen that the active material bound to the column and was eluted with the N-acetyl-D-glucosamine, whereas much of the contaminating material passed through the column during sample application and wash.

4. S-Sepharose Fast Flow Cation Exchange Chromatography

Fractions 211-225 from the wheat germ agglutinin-agarose chromatography shown in Figure 3 and equivalent fractions from the second run were pooled (375 ml) and dialyzed against 25 mM sodium formate, pH 4.2. To minimize the time of exposure to low pH, the dialysis was done over a period of 8 h, against 5 L of buffer, with four changes being made during the 8 h period. At the end of this dialysis period, the sample volume was 480 ml and the pH and conductivity of the sample were close to those of the dialysis buffer. Precipitated material appeared in the sample during dialysis. This was removed by centrifugation at 22,000 x g for 30 min, and the supernatant from the centrifuged sample was applied to a S-Sepharose Fast Flow cation exchange column (3.3 x 10.25 cm; resin from Pharmacia) which had been equilibrated in the sodium formate buffer. Flow rate was 465 ml/h and fractions of 14.2 ml were collected. After sample application, the column was washed with 240 ml of column buffer and elution of bound material was carried out by applying a gradient of 0-750 mM NaCl (NaCl dissolved in column buffer; total gradient volume 2200 ml), beginning at fraction ~45 in Figure 4. The elution profile is shown in Figure 4. Collected fractions were adjusted to pH 7-7.4 by addition of 200 µl of 0.97 M Tris base. The cpm in Figure 4 again refer to the results obtained in the MC/9 biological assay; portions of the indicated

fractions were dialyzed against phosphate-buffered saline, and 20 µl placed into the assay. It can be seen in Figure 4 that the majority of biologically active material passed through the column unbound, whereas much of the contaminating material bound and was eluted in the salt gradient.

5. Chromatography Using Silica-Bound Hydrocarbon Resin

Fractions 4-40 from the S-Sepharose column of Figure 4 were pooled (540 ml). 450 ml of the pool was combined with an equal volume of buffer B (100 mM ammonium acetate, pH 6:isopropanol; 25:75) and applied at a flow rate of 540 ml/h to a C_4 column (Vydac Proteins C_4; 2.4 x 2 cm) equilibrated with buffer A (60 mM ammonium acetate, pH 6:isopropanol; 62.5:37.5). After sample application, the flow rate was reduced to 154 ml/h and the column was washed with 200 ml of buffer A. A linear gradient from buffer A to buffer B (total volume 140 ml) was then applied, and fractions of 9.1 ml were collected. Portions of the pool from S-Sepharose chromatography, the C_4 column starting sample, runthrough pool, and wash pool were brought to 40 µg/ml bovine serum albumin by addition of an appropriate volume of a 1 mg/ml stock solution, and dialyzed against phosphate-buffered saline in preparation for biological assay. Similarly, 40 µl aliquots of the gradient fractions were combined with 360 µl of phosphate-buffered saline containing 16 µg bovine serum albumin, and this was followed by dialysis against phosphate-buffered saline in preparation for biological assay. These various fractions were assayed by the MC/9 assay (6.3 µl aliquots of the prepared gradient fractions; cpm in Figure 5). The assay results also indicated that about 75% of the recovered activity was in the runthrough and wash fractions, and 25% in the gradient fractions indicated in Figure 5. SDS-PAGE

[Laemmli, Nature, 227, 680-685 (1970); stacking gels contained 4% (w/v) acrylamide and separating gels contained 12.5% (w/v) acrylamide] of aliquots of various fractions is shown in Figure 6. For the gel shown, sample aliquots were dried under vacuum and then redissolved using 20 µl sample treatment buffer (nonreducing, i.e., without 2-mercaptoethanol) and boiled for 5 min prior to loading onto the gel. Lanes A and B represent column starting material (75 µl out of 890 ml) and column runthrough (75 µl out of 880 ml), respectively; the numbered marks at the left of the Figure represent migration positions (reduced) of markers having molecular weights of 10^3 times the indicated numbers, where the markers are phosphorylase b (M_r of 97,400), bovine serum albumin (M_r of 66,200), ovalbumin (M_r of 42,700), carbonic anhydrase (M_r of 31,000), soybean trypsin inhibitor (M_r of 21,500), and lysozyme (M_r of 14,400); lanes 4-9 represent the corresponding fractions collected during application of the gradient (60 µl out of 9.1 ml). The gel was silver-stained [Morrissey, Anal. Biochem., 117, 307-310 (1981)]. It can be seen by comparing lanes A and B that the majority of stainable material passes through the column. The stained material in fractions 4-6 in the regions just above and below the M_r 31,000 standard position coincides with the biological activity detected in the gradient fractions (Figure 5) and represents the biologically active material. It should be noted that this material is visualized in lanes 4-6, but not in lanes A and/or B, because a much larger proportion of the total volume (0.66% of the total for fractions 4-6 versus 0.0084% of the total for lanes A and B) was loaded for the former. Fractions 4-6 from this column were pooled.

As mentioned above, roughly 75% of the recovered activity ran through the C_4 column of

Figure 5. This material was rechromatographed using C_4 resin essentially as described above, except that a larger column (1.4 x 7.8 cm) and slower flow rate (50-60 ml/h throughout) were used. Roughly 50% of recovered activity was in the runthrough, and 50% in gradient fractions showing similar appearance on SDS-PAGE to that of the active gradient fractions in Figure 6. Active fractions were pooled (29 ml).

An analytical C_4 column was also performed essentially as stated above and the fractions were assayed in both bioassays. As indicated in Figure 7 of the fractions from this analytical column, both the MC/9 and HPP-CFC bioactivities co-elute. SDS-PAGE analysis (Figure 8) reveals the presence of the M_r ~31,000 protein in the column fractions which contain biological activity in both assays.

Active material in the second (relatively minor) activity peak seen in S-Sepharose chromatography (e.g. Figure 4, fractions 62-72, early fractions in the salt gradient) has also been purified by C_4 chromatography. It exhibited the same behavior on SDS-PAGE and had the same N-terminal amino acid sequence (see Example 2D) as the material obtained by C_4 chromatography of the S-Sepharose runthrough fractions.

6. Purification Summary

A summary of the purification steps described in 1-5 above is given in Table 2.

Table 2

Summary of Purification of Mammalian SCF

Step	Volume (ml)	Total Protein (mg)[5]
Conditioned medium	335,700	13,475
DEAE cellulose[1]	2,900	2,164
ACA-54	550	1,513
Wheat germ agglutinin-agarose[2]	375	431
S-Sepharose	540[4]	10
C_4 resin[3]	57.3	0.25-0.40[6]

1. Values given represent sums of the values for the different batches described in the text.
2. As described above in this Example, precipitated material which appeared during dialysis of this sample in preparation for S-Sepharose chromatography was removed by centrifugation. The sample after centrifugation (480 ml) contained 264 mg of total protein.
3. Combination of the active gradient fractions from the two C_4 columns run in sequence as described.
4. Only 450 ml of this material was used for the following step (this Example, above).
5. Determined by the method of Bradford (supra, 1976) except where indicated otherwise.
6. Estimate, based on intensity of silver-staining after SDS-PAGE, and on amino acid composition analysis as described in section K of Example 2.

D. SDS-PAGE and Glycosidase Treatments

SDS-PAGE of pooled gradient fractions from the two large scale C_4 column runs are shown in Figure 9. Sixty µl of the pool for the first C_4 column was loaded (lane 1), and 40 µl of the pool for the second C_4 column (lane 2). These gel lanes were silver-stained.

Molecular weight markers were as described for Figure 6. As mentioned, the diffusely-migrating material above and below the M_r 31,000 marker position represents the biologically active material; the apparent heterogeneity is largely due to heterogeneity in glycosylation.

To characterize the glycosylation, purified material was iodinated with ^{125}I, treated with a variety of glycosidases, and analyzed by SDS-PAGE (reducing conditions) with autoradiography. Results are shown in Figure 9. Lanes 3 and 9, ^{125}I-labeled material without any glycosidase treatment. Lanes 4-8 represent ^{125}I-labeled material treated with glycosidases, as follows. Lane 4, neuraminidase. Lane 5, neuraminidase and O-glycanase. Lane 6, N-glycanase. Lane 7, neuraminidase and N-glycanase. Lane 8, neuraminidase, O-glycanase, and N-glycanase. Conditions were 5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 33 mM 2-mercaptoethanol, 10 mM Tris-HCl, pH 7-7.2, for 3 h at 37°C. Neuraminidase (from Arthrobacter ureafaciens; Calbiochem) was used at 0.23 units/ml final concentration. O-Glycanase (Genzyme; endo-alpha-N-acetyl-galactosaminidase) was used at 45 milliunits/ml. N-Glycanase (Genzyme; peptide:N-glycosidase F; peptide-N^4[N-acetyl-beta-glucosaminyl]asparagine amidase) was used at 10 units/ml.

Similar results to those of Figure 9 were obtained upon treatment of unlabeled purified SCF with glycosidases, and visualization of products by silver-staining after SDS-PAGE.

Where appropriate, various control incubations were carried out. These included: incubation in appropriate buffer, but without glycosidases, to verify that results were due to the glycosidase preparations added; incubation with glycosylated proteins (e.g.

glycosylated recombinant human erythropoietin) known to be substrates for the glycosidases, to verify that the glycosidase enzymes used were active; and incubation with glycosidases but no substrate, to verify that the glycosidases were not themselves contributing to or obscuring the visualized gel bands.

Glycosidase treatments were also carried out with endo-beta-N-acetylglucosamidase F (endo F; NEN Dupont) and with endo-beta-N-acetylglucosaminidase H (endo H; NEN Dupont), again with appropriate control incubations. Conditions of treatment with endo F were: boiling 3 min in the presence of 1% (w/v) SDS, 100 mM 2-mercaptoethanol, 100 mM EDTA, 320 mM sodium phosphate, pH 6, followed by 3-fold dilution with the inclusion of Nonidet P-40 (1.17%, v/v, final concentration), sodium phosphate (200 mM, final concentration), and endo F (7 units/ml, final concentration). Conditions of endo H treatment were similar except that SDS concentration was 0.5% (w/v) and endo H was used at a concentration of 1 µg/ml. The results with endo F were the same as those with N-glycanase, whereas endo H had no effect on the purified SCF material.

A number of conclusions can be drawn from the glyosidase experiments described above. The various treatments with N-glycanase [which removes both complex and high-mannose N-linked carbohydrate (Tarentino et al., Biochemistry 24, 4665-4671) (1985)], endo F [which acts similarly to N-glycanase (Elder and Alexander, Proc. Natl. Acad. Sci. USA 79, 4540-4544 (1982)], endo H [which removes high-mannose and certain hybrid type N-linked carbohydrate (Tarentino et al., Methods Enzymol. 50C, 574-580 (1978)], neuraminidase (which removes sialic acid residues), and O-glycanase [which removes certain O-linked carbohydrates (Lambin et al., Biochem. Soc. Trans. 12, 599-600 (1984)], suggest that: both N-linked and O-linked carbohydrates

are present; most of the N-linked carbohydrate is of the complex type; and sialic acid is present, with at least some of it being part of the O-linked moieties. Some information about possible sites of N-linkage can be obtained from amino acid sequence data (Example 2). The fact that treatment with N-glycanase, endo F, and N-glycanase/neuraminidase can convert the heterogeneous material apparent by SDS-PAGE to faster-migrating forms which are much more homogeneous is consistent with the conclusion that all of the material represents the same polypeptide, with the heterogeneity being caused by heterogeneity in glycosylation. It is also noteworthy that the smallest forms obtained by the combined treatments with the various glycosidases are in the range of M_r 18,000-20,000, relative to the molecular weight markers used in the SDS-PAGE.

Confirmation that the diffusely-migrating material around the M_r 31,000 position on SDS-PAGE represents biologically active material all having the same basic polypeptide chain is given by the fact that amino acid sequence data derived from material migrating in this region (e.g., after electrophoretic transfer and cyanogen bromide treatment; Example 2) matches that demonstrated for the isolated gene whose expression by recombinant DNA means leads to biologically-active material (Example 4).

EXAMPLE 2

Amino Acid Sequence Analysis of Mammalian SCF

A. Reverse-phase High Performance Liquid Chromatography (HPLC) of Purified Protein

Approximately 5 µg of SCF purified as in Example 1 (concentration = 0.117 mg/ml) was subjected to

reverse-phase HPLC using a C_4 narrowbore column (Vydac, 300 Å widebore, 2 mm x 15 cm). The protein was eluted with a linear gradient from 97% mobile phase A (0.1% trifluoroacetic acid)/3% mobile phase B (90% acetonitrile in 0.1% trifluoroacetic acid) to 30% mobile phase A/70% mobile phase B in 70 min followed by isocratic elution for another 10 min at a flow rate of 0.2 ml per min. After subtraction of a buffer blank chromatogram, the SCF was apparent as a single symmetrical peak at a retention time of 70.05 min as shown in Figure 10. No major contaminating protein peaks could be detected under these conditions.

B. Sequencing of Electrophoretically-Transferred Protein Bands

SCF purified as in Example 1 (0.5-1.0 nmol) was treated as follows with N-glycanase, an enzyme which specifically cleaves the Asn-linked carbohydrate moieties covalently attached to proteins (see Example 1D). Six ml of the pooled material from fractions 4-6 of the C_4 column of Figure 5 was dried under vacuum. Then 150 µl of 14.25 mM CHAPS, 100 mM 2-mercaptoethanol, 335 mM sodium phosphate, pH 8.6 was added and incubation carried out for 95 min at 37°C. Next 300 µl of 74 mM sodium phosphate, 15 units/ml N-glycanase, pH 8.6 was added and incubation continued for 19 h. The sample was then run on a 9-18% SDS-polyacrylamide gradient gel (0.7 mm thickness, 20x20 cm). Protein bands in the gel were electrophoretically transferred onto polyvinyldifluoride (PVDF, Millipore Corp.) using 10 mM Caps buffer (pH 10.5) at a constant current of 0.5 Amp for 1 h [Matsudaira, J. Biol. Chem., 261, 10035-10038 (1987)]. The transferred protein bands were visualized by Coomassie Blue staining. Bands were present at M_r ~29,000-33,000 and M_r ~26,000, i.e., the

deglycosylation was only partial (refer to Example 1D, Figure 9); the former band represents undigested material and the latter represents material from which N-linked carbohydrate is removed. The bands were cut out and directly loaded (40% for M_r 29,000-33,000 protein and 80% for M_r 26,000 protein) onto a protein sequencer (Applied Biosystems Inc., model 477). Protein sequence analysis was performed using programs supplied by the manufacturer [Hewick et al., J. Biol. Chem., 256 7990-7997 (1981)] and the released phenylthiohydantoinyl amino acids were analyzed on-line using microbore C_{18} reverse-phase HPLC. Both bands gave no signals for 20-28 sequencing cycles, suggesting that both were unsequenceable by methodology using Edman chemistry. The background level on each sequencing run was between 1-7 pmol which was far below the protein amount present in the bands. These data suggested that protein in the bands was N-terminally blocked.

C. In-situ CNBr Cleavage of Electrophoretically-Transferred Protein and Sequencing

To confirm that the protein was in fact blocked, the membranes were removed from the sequencer (part B) and in situ cyanogen bromide (CNBr) cleavage of the blotted bands was carried out [CNBr (5%, w/v) in 70% formic acid for 1 h at 45°C] followed by drying and sequence analysis. Strong sequence signals were detected, representing internal peptides obtained from methionyl peptide bond cleavage by CNBr.

Both bands yielded identical mixed sequence signals listed below for the first five cycles.

	Amino Acids Identified
Cycle 1:	Asp; Glu; Val; Ile; Leu
Cycle 2:	Asp; Thr; Glu; Ala; Pro; Val
Cycle 3:	Asn; Ser; His; Pro; Leu
Cycle 4:	Asp; Asn; Ala; Pro; Leu
Cycle 5:	Ser; Tyr; Pro

Both bands also yielded similar signals up to 20 cycles. The initial yields were 40-115 pmol for the M_r 26,000 band and 40-150 pmol for the M_r 29,000-33,000 band. These values are comparable to the original molar amounts of protein loaded onto the sequencer. The results confirmed that protein bands corresponding to SCF contained a blocked N-terminus. Procedures used to obtain useful sequence information for N-terminally blocked proteins include: (a) deblocking the N-terminus (see section D); and (b) generating peptides by internal cleavages by CNBr (see Section E), by trypsin (see Section F), and by Staphylococcus aureus (strain V-8) protease (Glu-C) (see Section G). Sequence analysis can proceed after the blocked N-terminal amino acid is removed or the peptide fragments are isolated. Examples are described in detail below.

D. Sequence Analysis of BRL Stem Cell Factor Treated with Pyroglutamic Acid Aminopeptidase

The chemical nature of the blockage moiety present at the amino terminus of SCF was difficult to predict. Blockage can be post-translational in vivo [F. Wold, Ann. Rev. Biochem., 50, 783-814 (1981)] or may occur in vitro during purification. Two post-translational modifications are most commonly observed. Acetylation of certain N-terminal amino acids

such as Ala, Ser, etc. can occur, catalyzed by N-α-acetyl transferase. This can be confirmed by isolation and mass spectrometric analysis of an N-terminally blocked peptide. If the amino terminus of a protein is glutamine, deamidation of its gamma-amide can occur. Cyclization involving the gamma-carboxylate and the free N-terminus can then occur to yield pyroglutamate. To detect pyroglutamate, the enzyme pyroglutamate aminopeptidase can be used. This enzyme removes the pyroglutamate residue, leaving a free amino terminus starting at the second amino acid. Edman chemistry can then be used for sequencing.

SCF (purified as in Example 1; 400 pmol) in 50 mM sodium phosphate buffer (pH 7.6 containing dithiothreitol and EDTA) was incubated with 1.5 units of calf liver pyroglutamic acid aminopeptidase (pE-AP) for 16 h at 37°C. After reaction the mixture was directly loaded onto the protein sequencer. A major sequence could be identified through 46 cycles. The initial yield was about 40% and repetitive yield was 94.2%. The N-terminal sequence of SCF including the N-terminal pyroglutamic acid is:

pE-AP cleavage site

```
                +                                   10
pyroGlu-Glu-Ile-Cys-Arg-Asn-Pro-Val-Thr-Asp-Asn-Val-Lys-Asp-Ile-Thr-Lys-

          20                                  30
Leu-Val-Ala-Asn-Leu-Pro-Asn-Asp-Tyr-Met-Ile-Thr-Leu-Asn-Tyr-Val-

                          40
Ala-Gly-Met-Asp-Val-Leu-Pro-Ser-His-xxx-Trp-Leu-Arg-Asp-.....
```

xxx, not assigned at position 43

These results indicated that SCF contains pyroglutamic acid as its N-terminus.

E. Isolation and Sequence Analysis of CNBr Peptides

SCF purified as in Example 1 (20-28 µg; 1.0-1.5 nmol) was treated with N-glycanase as described in Example 1. Conversion to the M_r 26,000 material was complete in this case. The sample was dried and digested with CNBr in 70% formic acid (5%) for 18 h at room temperature. The digest was diluted with water, dried, and redissolved in 0.1% trifluoroacetic acid. CNBr peptides were separated by reverse-phase HPLC using a C_4 narrowbore column and elution conditions identical to those described in Section A of this Example. Several major peptide fractions were isolated and sequenced, and the results are summarized in the following:

Peptide	Retention Time (min)		Sequence[4]
CB-4	15.5		L-P-P---
CB-6[1]	22.1	a.	I-T-L-N-Y-V-A-G-(M)
		b.	V-A-S-D-T-S-D-C-V-L-S-_-_-L-G-P-E-K-D-S-R-V-S-V-(_)-K----
CB-8	28.0		D-V-L-P-S-H-C-W-L-R-D-(M)
CB-10	30.1		(containing sequence of CB-8)
CB-15[2]	43.0		E-E-N-A-P-K-N-V-K-E-S-L-K-K-P-T-R-(N)-F-T-P-E-E-F-F-S-I-F-D[3]-R-S-I-D-A------
CB-14 and CB-16	37.3		Both peptides contain identical sequence to CB-15

1. Amino acids were not detected at positions 12, 13 and 25. Peptide b was not sequenced to the end.
2. (N) in CB-15 was not detected; it was inferred based on the potential N-linked glycosylation site. The peptide was not sequenced to the end.
3. Designates site where Asn may have been converted into Asp upon N-glycanase removal of N-linked sugar.
4. Single letter code was used: A,Ala; C,Cys; D,Asp; E,Glu; F,Phe; G,Gly; H,His; I,Ile; K,Lys; L,Leu; M,Met; N,Asn; P,Pro; Q,Gln; R,Arg; S,Ser; T,Thr; V,Val; W,Trp; and Y,Tyr.

F. Isolation and Sequencing of BRL Stem Cell Factor Tryptic Fragments

SCF purified as in Example 1 (20 µg in 150 µl 0.1 M ammonium bicarbonate) was digested with 1 µg of trypsin at 37°C for 3.5 h. The digest was immediately run on reverse-phase narrow bore C_4 HPLC using elution conditions identical to those described in Section A of this Example. All eluted peptide peaks had retention times different from that of undigested SCF (Section A). The sequence analyses of the isolated peptides are shown below:

Peptide	Retention Time (min)	Sequence
T-1	7.1	E-S-L-K-K-P-E-T-R
T-2[1]	28.1	V-S-V-(_)-K
T-3	32.4	I-V-D-D-L-V-A-A-M-E-E-N-A-P-K
T-4[2]	40.0	N-F-T-P-E-E-F-F-S-I-F-(_)-R
T-5[3]	46.4	a. L-V-A-N-L-P-N-D-Y-M-I-T-L-N-Y-V-A-G-M-D-V-L-P-S-H-C-W-L-R
		b. S-I-D-A-F-K-D-F-M-V-A-S-D-T-S-D-C-V-L-S-(_)-(_)-L-G----
T-7[4]	72.8	E-S-L-K-K-P-E-T-R-(N)-F-T-P-E-E-F-F-S-I-F-(_)-R
T-8	73.6	E-S-L-K-K-P-E-T-R-N-F-T-P-E-E-F-F-S-I-F-D-R

1. Amino acid at position 4 was not assigned.
2. Amino acid at position 12 was not assigned.
3. Amino acids at positions 20 and 21 in 6 of peptide T-5 were not identified; they were tentatively assigned as O-linked sugar attachment sites.
4. Amino acid at position 10 was not detected; it was inferred as Asn based on the potential N-linked glycosylation site. Amino acid at position 21 was not detected.

G. Isolation and Sequencing of BRL Stem Cell Factor Peptides after S. aureus Glu-C Protease Cleavage

SCF purified as in Example 1 (20 µg in 150 µl 0.1 M ammonium bicarbonate) was subjected to Glu-C protease cleavage at a protease-to-substrate ratio of 1:20. The digestion was accomplished at 37°C for 18 h. The digest was immediately separated by reverse-phase narrowbore C_4 HPLC. Five major peptide fractions were collected and sequenced as described below:

Peptides	Retention Time (min)	Sequence
S-1	5.1	N-A-P-K-N-V-K-E
S-2[1]	27.7	S-R-V-S-V-(_)-K-P-F-M-L-P-P-V-A-(A)
S-3[2]	46.3	No sequence detected
S-5[3]	71.0	S-L-K-K-P-E-T-R-N-F-T-P-E-E-F-F-S-I-F-(N)-R-S-I-D-A-F-K-D-F-M-V-A-S-D
S-6[3]	72.6	S-L-K-K-P-E-T-R-N-F-T-P-E-E-F-F-S-I-F-(N)-R-S-I-D-A-F-K-D-F-M-V-A-S-D-T-S-D

1. Amino acid at position 6 of S-2 peptide was not assigned; this could be an O-linked sugar attachment site. The Ala at position 16 of S-2 peptide was detected in low yield.
2. Peptide S-3 could be the N-terminally blocked peptide derived from the N-terminus of SCF.
3. N in parentheses was assigned as a potential N-linked sugar attachment site.

H. Sequence Analysis of BRL Stem Cell Factor after BNPS-skatole Cleavage

SCF (2 µg) in 10 mM ammonium bicarbonate was dried to completeness by vacuum centrifugation and then redissolved in 100 ul of glacial acetic acid. A 10-20 fold molar excess of BNPS-skatole was added to the solution and the mixture was incubated at 50°C for 60 min. The reaction mixture was then dried by vacuum centrifugation. The dried residue was extracted with 100 µl of water and again with 50 µl of water. The combined extracts were then subjected to sequence analysis as described above. The following sequence was detected:

1 10

Leu-Arg-Asp-Met-Val-Thr-His-Leu-Ser-Val-Ser-Leu-Thr-Thr-Leu-Leu-

20 30

Asp-Lys-Phe-Ser-Asn-Ile-Ser-Glu-Gly-Leu-Ser-(Asn)-Tyr-Ser-Ile-Ile-

40

Asp-Lys-Leu-Gly-Lys-Ile-Val-Asp----

Position 28 was not positively assigned; it was assigned as Asn based on the potential N-linked glycosylation site.

I. C-Terminal Amino Acid Determination of BRL Stem Cell Factor

An aliquot of SCF protein (500 pmol) was buffer-exchanged into 10 mM sodium acetate, pH 4.0 (final volume of 90 µl) and Brij-35 was added to 0.05% (w/v). A 5 µl aliquot was taken for quantitation of protein. Forty µl of the sample was diluted to 100 µl with the buffer described above. Carboxypeptidase P (from Penicillium janthinellum) was added at an enzyme-to-substrate ratio of 1:200. The digestion proceeded at 25°C and 20 µl aliquots were taken at 0, 15, 30, 60 and

120 min. The digestion was terminated at each time point by adding trifluoroacetic acid to a final concentration of 5%. The samples were dried and the released amino acids were derivatized by reaction with Dabsyl chloride (dimethylaminoazobenzenesulfonyl chloride) in 0.2 M $NaHCO_3$ (pH 9.0) at 70°C for 12 min [Chang et al., Methods Enzymol., 90, 41-48 (1983)]. The derivatized amino acids (one-sixth of each sample) were analyzed by narrowbore reverse-phase HPLC with a modification of the procedure of Chang et al. [Techniques in Protein Chemistry, T. Hugli ed., Acad. Press, NY (1989), pp. 305-311]. Quantitative composition results at each time point were obtained by comparison to derivatized amino acid standards (1 pmol). At 0 time, contaminating glycine was detected. Alanine was the only amino acid that increased with incubation time. After 2 h incubation, Ala was detected at a total amount of 25 pmol, equivalent to 0.66 mole of Ala released per mole of protein. This result indicated that the natural mammalian SCF molecule contains Ala as its carboxyl terminus, consistent with the sequence analysis of a C-terminal peptide, S-2, which contains C-terminal Ala. This conclusion is also consistent with the known specificity of carboxypeptidase P [Lu et al., J. Chromatog. 447, 351-364 (1988)]. For example, cleavage ceases if the sequence Pro-Val is encountered. Peptide S-2 has the sequence S-R-V-S-V-(T)-K-P-F-M-L-P-P-V-A-(A) and was deduced to be the C-terminal peptide of SCF (see Section J in this Example). The C-terminal sequence of ---P-V-A-(A) restricts the protease cleavage to alanine only. The amino acid composition of peptide S-2 indicates the presence of 1 Thr, 2 Ser, 3 Pro, 2 Ala, 3 Val, 1 Met, 1 Leu, 1 Phe, 1 Lys, and 1 Arg, totalling 16 residues. The detection of 2 Ala residues indicates that there may be two Ala residues at the C-terminus of this peptide

(see table in Section G). Thus the BRL SCF terminates at Ala 164 or Ala 165.

J. Sequence of SCF

By combining the results obtained from sequence analysis of (1) intact stem cell factor after removing its N-terminal pyroglutamic acid, (2) the CNBr peptides, (3) the trypsin peptides, and (4) the Glu-C peptidase fragments, an N-terminal sequence and a C-terminal sequence were deduced (Figure 11). The N-terminal sequence starts at pyroglutamic acid and ends at Met-48. The C-terminal sequence contains 84/85 amino acids (position 82 to 164/165). The sequence from position 49 to 81 was not detected in any of the peptides isolated. However, a sequence was detected for a large peptide after BNPS-skatole cleavage of BRL SCF as described in Section H of this Example. From these additional data, as well as DNA sequence obtained from rat SCF (Example 3) the N- and C-terminal sequences can be aligned and the overall sequence delineated as shown in Figure 11. The N-terminus of the molecule is pyroglutamic acid and the C-terminus is alanine as confirmed by pyroglutamate aminopeptidase digestion and carboxypeptidase P digestion, respectively.

From the sequence data, it is concluded that Asn-72 is glycosylated; Asn-109 and Asn-120 are probably glycosylated in some molecules but not in others. Asn-65 could be detected during sequence analysis and therefore may only be partially glycosylated, if at all. Ser-142, Thr-143 and Thr-155, predicted from DNA sequence, could not be detected during amino acid sequence analysis and therefore could be sites of O-linked carbohydrate attachment. These potential carbohydrate attachment sites are indicated in Figure 11; N-linked carbohydrate is indicated by solid

bold lettering; O-linked carbohydrate is indicated by open bold lettering.

K. Amino Acid Compositional Analysis of BRL Stem Cell Factor

Material from the C_4 column of Figure 7 was prepared for amino acid composition analysis by concentration and buffer exchange into 50 mM ammonium bicarbonate.

Two 70 µl samples were separately hydrolyzed in 6 N HCl containing 0.1% phenol and 0.05% 2-mercaptoethanol at 110°C in vacuo for 24 h. The hydrolysates were dried, reconstituted into sodium citrate buffer, and analyzed using ion exchange chromatography (Beckman Model 6300 amino acid analyzer). The results are shown in Table 3. Using 164 amino acids (from the protein sequencing data) to calculate amino acid composition gives a better match to predicted values than using 193 amino acids (as deduced from PCR-derived DNA sequencing data, Figure 14C).

Table 3

Quantitative Amino Acid Composition of Mammalian Derived SCF

Amino Acid	Amino Acid Composition Moles per mole of protein[1] Run #1	Run #2	Predicted Residues per molecule[2] (A)	(B)
Asx	24.46	24.26	25	28
Thr	10.37	10.43	11	12
Ser	14.52	14.30	16	24
Glx	11.44	11.37	10	10
Pro	10.90	10.85	9	10
Gly	5.81	6.20	4	5
Ala	8.62	8.35	7/8	8
Cys	nd	nd	4	5
Val	14.03	13.96	15	15
Met	4.05	3.99	6	7
Ile	8.31	8.33	9	10
Leu	17.02	16.97	16	19
Tyr	2.86	2.84	3	7
Phe	7.96	7.92	8	8
His	2.11	2.11	2	3
Lys	10.35	11.28	12	14
Trp	nd	nd	1	1
Arg	4.93	4.99	5	6
Total	158	158	164/165	193
Calculated molecular weight			18,424[3]	

1. Based on 158 residues from protein sequence analysis (excluding Cys and Trp).
2. Theoretical values calculated from protein sequence data (A) or from DNA sequence data (B).
3. Based on 1-164 sequence.

Inclusion of a known amount of an internal standard in the amino acid composition analyses also allowed quantitation of protein in the sample; a value of 0.117 mg/ml was obtained for the sample analyzed.

EXAMPLE 3

Cloning of the Genes for Rat and Human SCF

A. Amplification and Sequencing of Rat SCF cDNA Fragments

Determination of the amino acid sequence of fragments of the rat SCF protein made it possible to design mixed sequence oligonucleotides specific for rat SCF. The oligonucleotides were used as hybridization probes to screen rat cDNA and genomic libraries and as primers in attempts to amplify portions of the cDNA using polymerase chain reaction (PCR) strategies ([Mullis et al., Methods in Enzymol. 155, 335-350 (1987)]. The oligodeoxynucleotides were synthesized by the phosphoramidite method [Beaucage, et al., Tetrahedron Lett., 22, 1859-1862 (1981); McBride, et al., Tetrahedron Lett., 24, 245-248 (1983)]; their sequences are depicted in Figure 12A. The letters represent A, adenine; T, thymine, C, cytosine; G, guanine; I, inosine. The * in Figure 12A represents oligonucleotides which contain restriction endonuclease recognition sequences. The sequences are written 5'→3'.

A rat genomic library, a rat liver cDNA library, and two BRL cDNA libraries were screened using ^{32}P-labelled mixed oligonucleotide probes, 219-21 and 219-22 (Figure 12A), whose sequences were based on amino acid sequence obtained as in Example 2. No SCF clones were isolated in these experiments using standard methods of cDNA cloning [Maniatis, et al., Molecular Cloning, Cold Spring Harbor 212-246 (1982)].

An alternate approach which did result in the isolation of SCF nucleic acid sequences involved the use of PCR techniques. In this methodology, the region of DNA encompassed by two DNA primers is amplified selectively in vitro by multiple cycles of replication

catalysed by a suitable DNA polymerase (such as TaqI DNA polymerase) in the presence of deoxynucleoside triphosphates in a thermo cycler. The specificity of PCR amplification is based on two oligonucleotide primers which flank the DNA segment to be amplified and hybridize to opposite strands. PCR with double-sided specificity for a particular DNA region in a complex mixture is accomplished by use of two primers with sequences sufficiently specific to that region. PCR with single-sided specificity utilizes one region-specific primer and a second primer which can prime at target sites present on many or all of the DNA molecules in a particular mixture [Loh et al., Science, 243, 217-220 (1989)].

The DNA products of successful PCR amplification reactions are sources of DNA sequence information [Gyllensten, Biotechniques, 7, 700-708 (1989)] and can be used to make labeled hybridization probes possessing greater length and higher specificity than oligonucleotide probes. PCR products can also be designed, with appropriate primer sequences, to be cloned into plasmid vectors which allow the expression of the encoded peptide product.

The basic strategy for obtaining the DNA sequence of the rat SCF cDNA is outlined in Figure 13A. The small arrows indicate PCR amplifications and the thick arrows indicate DNA sequencing reactions. PCRs 90.6 and 96.2, in conjunction with DNA sequencing, were used to obtain partial nucleic acid sequence for the rat SCF cDNA. The primers used in these PCRs were mixed oligonucleotides based on amino acid sequence depicted in Figure 11. Using the sequence information obtained from PCRs 90.6 and 96.2, unique sequence primers (224-27 and 224-28, Figure 12A) were made and used in subsequent amplifications and sequencing reactions. DNA containing

the 5' end of the cDNA was obtained in PCRs 90.3, 96.6, and 625.1 using single-sided specificity PCR. Additional DNA sequence near the C-terminus of SCF protein was obtained in PCR 90.4. DNA sequence for the remainder of the coding region of rat SCF cDNA was obtained from PCR products 630.1, 630.2, 84.1 and 84.2 as described below in section C of this Example. The techniques used in obtaining the rat SCF cDNA are described below.

RNA was prepared from BRL cells as described by Okayama et al. [Methods Enzymol., 154, 3-28 (1987)]. PolyA+ RNA was isolated using an oligo(dT) cellulose column as described by Jacobson in [Methods in Enzymology, volume 152, 254-261 (1987)].

First-strand cDNA was synthesized using 1 µg of BRL polyA+ RNA as template and $(dT)_{12-18}$ as primer according to the protocol supplied with the enzyme, Mo-MLV reverse transcriptase (Bethesda Research Laboratories). RNA strand degradation was performed using 0.14 M NaOH at 84°C for 10 min or incubation in a boiling water bath for 5 min. Excess ammonium acetate was added to neutralize the solution, and the cDNA was first extracted with phenol/chloroform, then extracted with chloroform/iso-amyl alcohol, and precipitated with ethanol. To make possible the use of oligo(dC)-related primers in PCRs with single-sided specificity, a poly(dG) tail was added to the 3' terminus of an aliquot of the first-strand cDNA with terminal transferase from calf thymus (Boeringer Mannheim) as previously described [Deng et al., Methods Enzymol., 100, 96-103 (1983)].

Unless otherwise noted in the descriptions which follow, the denaturation step in each PCR cycle was set at 94°C, 1 min; and elongation was at 72°C for 3 or 4 min. The temperature and duration of annealing was variable from PCR to PCR, often representing a compromise based on the estimated requirements of

several different PCRs being carried out simultaneously. When primer concentrations were reduced to lessen the accumulation of primer artifacts [Watson, Amplifications, 2, 56 (1989)], longer annealing times were indicated; when PCR product concentration was high, shorter annealing times and higher primer concentrations were used to increase yield. A major factor in determining the annealing temperature was the estimated T_d of primer-target association [Suggs et al., in Developmental Biology Using Purified Genes eds. Brown, D.D. and Fox, C.F. (Academic, New York) pp. 683-693 (1981)]. The enzymes used in the amplifications were obtained from either of three manufacturers: Stratagene, Promega, or Perkin-Elmer Cetus. The reaction compounds were used as suggested by the manufacturer. The amplifications were performed in either a Coy Tempcycle or a Perkin-Elmer Cetus DNA thermocycler.

Amplification of SCF cDNA fragments was usually assayed by agarose gel electrophoresis in the presence of ethidium bromide and visualization by fluorescence of DNA bands stimulated by ultraviolet irradiation. In some cases where small fragments were anticipated, PCR products were analyzed by polyacrylamide gel electrophoresis. Confirmation that the observed bands represented SCF cDNA fragments was obtained by observation of appropriate DNA bands upon subsequent amplification with one or more internally-nested primers. Final confirmation was by dideoxy sequencing [Sanger et al., Proc. Natl. Acad. Sci. USA, 74, 5463-5467 (1977)] of the PCR product and comparison of the predicted translation products with SCF peptide sequence information.

In the initial PCR experiments, mixed oligonucleotides based on SCF protein sequence were used [Gould, Proc. Natl. Acad. Sci. USA, 86, 1934-1938

(1989)]. Below are descriptions of the PCR amplifications that were used to obtain DNA sequence information for the rat cDNA encoding amino acids -25 to 162.

In PCR 90.6, BRL cDNA was amplified with 4 pmol each of 222-11 and 223-6 in a reaction volume of 20 µl. An aliquot of the product of PCR 90.6 was electrophoresed on an agarose gel and a band of about the expected size was observed. One µl of the PCR 90.6 product was amplified further with 20 pmol each of primers 222-11 and 223-6 in 50 µl for 15 cycles, annealing at 45°C. A portion of this product was then subjected to 25 cycles of amplification in the presence of primers 222-11 and 219-25 (PCR 96.2), yielding a single major product band upon agarose gel electrophoresis. Asymmetric amplification of the product of PCR 96.2 with the same two primers produced a template which was successfully sequenced. Further selective amplification of SCF sequences in the product of 96.2 was performed by PCR amplification of the product in the presence of 222-11 and nested primer 219-21. The product of this PCR was used as a template for asymmetric amplification and radiolabelled probe production (PCR2).

To isolate the 5' end of the rat SCF cDNA, primers containing $(dC)_n$ sequences, complimentary to the poly(dG) tails of the cDNA, were utilized as non-specific primers. PCR 90.3 contained $(dC)_{12}$ (10 pmol) and 223-6 (4 pmol) as primers and BRL cDNA as template. The reaction product acted like a very high molecular weight aggregate, remaining close to the loading well in agarose gel electrophoresis. One µl of the product solution was further amplified in the presence of 25 pmol of $(dC)_{12}$ and 10 pmol 223-6 in a volume of 25 ul for 15 cycles, annealing at 45°C. One-half µl of this product was then amplified for 25 cycles

with internally nested primer 219-25 and 201-7 (PCR 96.6). The sequence of 201-7 is shown in Figure 12C. No bands were observed by agarose gel electrophoresis. Another 25 cycles of PCR, annealing at 40°C, were performed, after which one prominent band was observed. Southern blotting was carried out and a single prominent hybridizing band was observed. An additional 20 cycles of PCR (625.1), annealing at 45°C, were performed using 201-7 and nested primer 224-27. Sequencing was performed after asymmetric amplification by PCR, yielding sequence which extended past the putative amino terminus of the presumed signal peptide coding sequence of pre-SCF. This sequence was used to design oligonucleotide primer 227-29 containing the 5' end of the coding region of the rat SCF cDNA. Similarly, the 3' DNA sequence ending at amino acid 162 was obtained by sequencing PCR 90.4 (see Figure 13.A).

B. Cloning of the Rat Stem Cell Factor Genomic DNA

Probes made from PCR amplification of cDNA encoding rat SCF as described in section A above were used to screen a library containing rat genomic sequences (obtained from CLONTECH Laboratories, Inc.; catalog number RL1022 j). The library was constructed in the bacteriophage λ vector EMBL-3 SP6/T7 using DNA obtained from an adult male Sprague-Dawley rat. The library, as characterized by the supplier, contains 2.3 $x10^6$ independent clones with an average insert size of 16 kb.

PCRs were used to generate ^{32}P-labeled probes used in screening the genomic library. Probe PCR1 (Figure 13A) was prepared in a reaction which contained 16.7 μM ^{32}P[alpha]-dATP, 200 μM dCTP, 200 μM dGTP, 200 μM dTTP, reaction buffer supplied by Perkin Elmer Cetus, Taq polymerase (Perkin Elmer Cetus) at 0.05

units/ml, 0.5 μM 219-26, 0.05 μM 223-6 and 1 μl of template 90.1 containing the target sites for the two primers. Probe PCR 2 was made using similar reaction conditions except that the primers and template were changed. Probe PCR 2 was made using 0.5 μM 222-11, 0.05 μM 219-21 and 1 μl of a template derived from PCR 96.2.

Approximately 10^6 bacteriophage were plated as described in Maniatis et al. [supra (1982)]. The plaques were transferred to GeneScreen Plus™ filters (22cm x 22cm; NEN/DuPont) which were denatured, neutralized and dried as described in a protocol from the manufacturer. Two filter transfers were performed for each plate.

The filters were prehybridized in 1M NaCl, 1% SDS, 0.1% bovine serum albumin, 0.1% ficoll, 0.1% polyvinylpyrrolidone (hybridization solution) for approximately 16 h at 65°C and stored at -20°C. The filters were transfered to fresh hybridization solution containing ^{32}P-labeled PCR 1 probe at 1.2 x 10^5 cpm/ml and hybridized for 14 h at 65°C. The filters were washed in 0.9 M NaCl, 0.09 M sodium citrate, 0.1% SDS, pH 7.2 (wash solution) for 2 h at room temperature followed by a second wash in fresh wash solution for 30 min at 65°C. Bacteriophage clones from the areas of the plates corresponding to radioactive spots on autoradiograms were removed from the plates and rescreened with probes PCR1 and PCR2.

DNA from positive clones was digested with restriction endonucleases BamHI, SphI or SstI, and the resulting fragments were subcloned into pUC119 and subsequently sequenced. The strategy for sequencing the rat genomic SCF DNA is shown schematically in Figure 14A. In this figure, the line drawing at the top represents the region of rat genomic DNA encoding SCF. The gaps in the line indicate regions that have not been

sequenced. The large boxes represent exons for coding regions of the SCF gene with the corresponding encoded amino acids indicated above each box. The arrows represent the individual regions that were sequenced and used to assemble the consensus sequence for the rat SCF gene. The sequence for rat SCF gene is shown in Figure 14B.

Using PCR 1 probe to screen the rat genomic library, clones corresponding to exons encoding amino acids 19 to 176 of SCF were isolated. To obtain clones for exons upstream of the coding region for amino acid 19, the library was screened using oligonucleotide probe 228-30. The same set of filters used previously with probe PCR 1 were prehybridized as before and hybridized in hybridization solution containing ^{32}P-labeled oligonucleotide 228-30 (0.03 picomole/ml) at 50°C for 16 h. The filters were washed in wash solution at room temperature for 30 min followed by a second wash in fresh wash solution at 45°C for 15 min. Bacteriophage clones from the areas of the plates corresponding to radioactive spots on autoradiograms were removed from the plates and rescreened with probe 228-30. DNA from positive clones was digested with restriction endonucleases and subcloned as before. Using probe 228-30, clones corresponding to the exon encoding amino acids -20 to 18 were obtained.

Several attempts were made to isolate clones corresponding to the exon(s) containing the 5'-untranslated region and the coding region for amino acids -25 to -21. No clones for this region of the rat SCF gene have been isolated.

C. Cloning Rat cDNA for Expression in Mammalian Cells

Mammalian cell expression systems were devised to ascertain whether an active polypeptide product of

rat SCF could be expressed in and secreted by mammalian cells. Expression systems were designed to express truncated versions of rat SCF (SCF^{1-162} and SCF^{1-164}) and a protein (SCF^{1-193}) predicted from the translation of the gene sequence in Fig. 14C.

The expression vector used in these studies was a shuttle vector containing pUC119, SV40 and HTLVI sequences. The vector was designed to allow autonomous replication in both E. coli and mammalian cells and to express inserted exogenous DNA under the control of viral DNA sequences. This vector, designated V19.8, harbored in E. coli DH5, is deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. (ATCC# 68124). This vector is a derivative of pSVDM19 described in Souza U.S. Patent 4,810,643 hereby incorporated by reference.

The cDNA for rat SCF^{1-162} was inserted into plasmid vector V19.8. The cDNA sequence is shown in Figure 14C. The cDNA that was used in this construction was synthesized in PCR reactions 630.1 and 630.2, as shown in Figure 13A. These PCRs represent independent amplifications and utilized synthetic oligonucleotide primers 227-29 and 227-30. The sequence for these primers was obtained from PCR generated cDNA as described in section A of this Example. The reactions, 50 µl in volume, consisted of 1x reaction buffer (from a Perkin Elmer Cetus kit), 250 µM dATP, 250 µM dCTP, 250 µM dGTP, and 250 µM dTTP, 200 ng oligo(dT)-primed cDNA, 1 picomole of 227-29, 1 picomole of 227-30, and 2.5 units of Taq polymerase (Perkin Elmer Cetus). The cDNA was amplified for 10 cycles using a denaturation temperature of 94°C for 1 min, an annealing temperature of 37°C for 2 min, and an elongation temperature of 72°C for 1 min. After these initial rounds of PCR amplification, 10 picomoles of 227-29 and 10 picomoles of 227-30 were added to each reaction. Amplifications

were continued for 30 cycles under the same conditions with the exception that the annealing temperature was changed to 55°C. The products of the PCR were digested with restriction endonucleases HindIII and SstII. V19.8 was similarly digested with HindIII and SstII, and in one instance, the digested plasmid vector was treated with calf intestinal alkaline phosphatase; in other instances, the large fragment from the digestion was isolated from an agarose gel. The cDNA was ligated to V19.8 using T4 polynucleotide ligase. The ligation products were transformed into competent E. coli strain DH5 as described [Okayama, et. al., supra (1987)]. DNA prepared from individual bacterial clones was sequenced by the Sanger dideoxy method. Figure 17 shows a construct of V19.8 SCF. These plasmids were used to transfect mammalian cells as described in Example 4 and Example 5.

The expression vector for rat SCF^{1-164} was constructed using a strategy similar to that used for SCF^{1-162} in which cDNA was synthesized using PCR amplification and subsequently inserted into V19.8. The cDNA used in the constructions was synthesized in PCR amplifications with V19.8 containing SCF^{1-162} cDNA (V19.8:SCF^{1-162}) as template, 227-29 as the primer for the 5'-end of the gene and 237-19 as the primer for the 3'-end of the gene. Duplicate reactions (50 ul) contained 1x reaction buffer, 250 uM each of dATP, dCTP, dGTP and dTTP, 2.5 units of Taq polymerase, 20 ng of V19.8:SCF^{1-162}, and 20 picomoles of each primer. The cDNA was amplified for 35 cycles using a denaturation temperature of 94°C for 1 min, an annealing temperature of 55°C for 2 min and an elongation temperature of 72°C for 2 min. The products of the amplifications were digested with restriction endonucleases HindIII and SstII and inserted into V19.8. The resulting vector contains the coding region for amino acids -25 to 164 of SCF followed by a termination codon.

The cDNA for a 193 amino acid form of rat SCF, (rat SCF^{1-193} is predicted from the translation of the DNA sequence in Figure 14C) was also inserted into plasmid vector V19.8 using a protocol similar to that used for the rat SCF^{1-162}. The cDNA that was used in this construction was synthesized in PCR reactions 84.1 and 84.2 (Figure 13A) utilizing oligonucleotides 227-29 and 230-25. The two reactions represent independent amplifications starting from different RNA preparations. The sequence for 227-29 was obtained via PCR reactions as described in section A of this Example and the sequence for primer 230-25 was obtained from rat genomic DNA (Figure 14B). The reactions, 50 µl in volume, consisted of 1x reaction buffer (from a Perkin Elmer Cetus kit), 250 µM dATP, 250 µM dCTP, 250 µM dGTP, and 250 µM dTTP, 200 ng oligo(dT)-primed cDNA, 10 picomoles of 227-29, 10 picomoles of 230-25, and 2.5 units of Taq polymerase (Perkin Elmer Cetus). The cDNA was amplified for 5 cycles using a denaturation temperature of 94°C for 1 1/2 minutes, an annealing temperature of 50°C for 2 min, and an elongation temperature of 72°C for 2 min. After these initial rounds, the amplifications were continued for 35 cycles under the same conditions with the exception that the annealing temperature was changed to 60°C. The products of the PCR amplification were digested with restriction endonucleases HindIII and SstII. V19.8 DNA was digested with HindIII and SstII and the large fragment from the digestion was isolated from an agarose gel. The cDNA was ligated to V19.8 using T4 polynucleotide ligase. The ligation products were transformed into competent E. coli strain DH5 and DNA prepared from individual bacterial clones was sequenced. These plasmids were used to transfect mammalian cells in Example 4.

D. Amplification and Sequencing of Human SCF cDNA PCR Products

The human SCF cDNA was obtained from a hepatoma cell line HepG2 (ATCC HB 8065) using PCR amplification as outlined in Figure 13B. The basic strategy was to amplify human cDNA by PCR with primers whose sequence was obtained from the rat SCF cDNA.

RNA was prepared as described by Maniatis et al. [supra (1982)]. PolyA+ RNA was prepared using oligo dT cellulose following manufacturers directions. (Collaborative Research Inc.).

First strand cDNA was prepared as described above for BRL cDNA, except that synthesis was primed with 2 μM oligonucleotide 228-28, shown in Figure 12C, which contains a short random sequence at the 3' end attached to a longer unique sequence. The unique-sequence portion of 228-28 provides a target site for amplification by PCR with primer 228-29 as non-specific primer. Human cDNA sequences related to at least part of the rat SCF sequence were amplified from the HepG2 cDNA by PCR using primers 227-29 and 228-29 (PCR 22.7, see Figure 13B; 15 cycles annealing at 60°C followed by 15 cycles annealing at 55°C). Agarose gel electrophoresis revealed no distinct bands, only a smear of apparently heterogeneously sized DNA. Further preferential amplification of sequences closely related to rat SCF cDNA was attempted by carrying out PCR with 1 μl of the PCR 22.7 product using internally nested rat SCF primer 222-11 and primer 228-29 (PCR 24.3; 20 cycles annealing at 55°C). Again only a heterogeneous smear of DNA product was observed on agarose gels. Double-sided specific amplification of the PCR 24.3 products with primers 222-11 and 227-30 (PCR 25.10; 20 cycles) gave rise to a single major product band of the same size as the corresponding rat SCF cDNA PCR product. Sequencing

of an asymmetric PCR product (PCR 33.1) DNA using 224-24 as sequencing primer yielded about 70 bases of human SCF sequences.

Similarly, amplification of 1 µl of the PCR 22.7 product, first with primers 224-25 and 228-29 (PCR 24.7, 20 cycles), then with primers 224-25 and 227-30 (PCR 41.11) generated one major band of the same size as the corresponding rat SCF product, and after asymmetric amplification (PCR 42.3) yielded a sequence which was highly homologous to the rat SCF sequence when 224-24 was used as sequencing primer. Unique sequence oligodeoxynucleotides targeted at the human SCF cDNA were synthesized and their sequences are given in Figure 12B.

To obtain the human counterpart of the rat SCF PCR-generated coding sequence which was used in expression and activity studies, a PCR with primers 227-29 and 227-30 was performed on 1 µl of PCR 22.7 product in a reaction volume of 50 µl (PCR 39.1). Amplification was performed in a Coy Tempcycler. Because the degree of mismatching between the human SCF cDNA and the rat SCF unique primer 227-30 was unknown, a low stringency of annealing (37°C) was used for the first three cycles; afterward annealing was at 55°C. A prominent band of the same size (about 590 bp) as the rat homologue appeared, and was further amplified by dilution of a small portion of PCR 39.1 product and PCR with the same primers (PCR 41.1). Because more than one band was observed in the products of PCR 41.1, further PCR with nested internal primers was performed in order to determine at least a portion of its sequence before cloning. After 23 cycles of PCR with primers 231-27 and 227-29 (PCR 51.2), a single, intense band was apparent. Asymmetric PCRs with primers 227-29 and 231-27 and sequencing confirmed the presence of the human SCF cDNA sequences. Cloning of the PCR 41.1 SCF

DNA into the expression vector V19.8 was performed as already described for the rat SCF 1-162 PCR fragments in Section C above. DNA from individual bacterial clones was sequenced by the Sanger dideoxy method.

E. Cloning of the Human Stem Cell Factor Genomic DNA

A PCR7 probe made from PCR amplification of cDNA, see Figure 13B, was used to screen a library containing human genomic sequences. A riboprobe complementary to a portion of human SCF cDNA, see below, was used to re-screen positive plaques. PCR 7 probe was prepared starting with the product of PCR 41.1 (see Figure 13B). The product of PCR 41.1 was further amplified with primers 227-29 and 227-30. The resulting 590 bp fragment was eluted from an agarose gel and reamplified with the same primers (PCR 58.1). The product of PCR 58.1 was diluted 1000-fold in a 50 µl reaction containing 10 pmoles 233-13 and amplified for 10 cycles. After the addition of 10 pmoles of 227-30 to the reaction, the PCR was continued for 20 cycles. An additional 80 pmoles of 233-13 was added and the reaction volume increased to 90 µl and the PCR was continued for 15 cycles. The reaction products were diluted 200-fold in a 50 µl reaction, 20 pmoles of 231-27 and 20 pmoles of 233-13 were added, and PCR was performed for 35 cycles using an annealing temperature of 48° in reaction 96.1. To produce ^{32}P-labeled PCR7, reaction conditions similar to those used to make PCR1 were used with the following exceptions: in a reaction volume of 50 µl, PCR 96.1 was diluted 100-fold; 5 pmoles of 231-27 was used as the sole primer; and 45 cycles of PCR were performed with denaturation at 94° for 1 minute, annealing at 48° for 2 minutes and elongation at 72° for 2 minutes.

The riboprobe, riboprobe 1, was a ^{32}P-labelled single-stranded RNA complementary to nucleotides 2-436 of the hSCF DNA sequence shown in Figure 15B. To construct the vector for the production of this probe, PCR 41.1 (Figure 13B) product DNA was digested with HindIII and EcoRI and cloned into the polylinker of the plasmid vector pGEM3 (Promega, Madison, Wisconsin). The recombinant pGEM3:hSCF plasmid DNA was then linearized by digestion with HindIII. ^{32}P-labeled riboprobe 1 was prepared from the linearized plasmid DNA by runoff transcription with T7 RNA polymerase according to the instructions provided by Promega. The reaction (3 µl) contained 250 ng of linearized plasmid DNA and 20 µM ^{32}P-rCTP (catalog #NEG-008H, New England Nuclear (NEN) with no additional unlabeled CTP.

The human genomic library was obtained from Stratagene (La Jolla, CA; catalog #:946203). The library was constructed in the bacteriophage Lambda Fix II vector using DNA prepared from a Caucasian male placenta. The library, as characterized by the supplier, contained $2x10^6$ primary plaques with an average insert size greater than 15 kb. Approximately 10^6 bacteriophage were plated as described in Maniatis, et al. [supra (1982)]. The plaques were transferred to Gene Screen Plus™ filters (22 cm^2; NEN/DuPont) according to the protocol from the manufacturer. Two filter transfers were performed for each plate.

The filters were prehybridized in 6XSSC (0.9 M NaCl, 0.09 M sodium citrate pH 7.5), 1% SDS at 60°C. The filters were hybridized in fresh 6XSSC, 1% SDS solution containing ^{32}P-labeled PCR 7 probe at $2x10^5$ cpm/ml and hybridized for 20 h at 62°C. The filters were washed in 6XSSC, 1% SDS for 16 h at 62°C. A bacteriophage plug was removed from an area of a plate which corresponded to radioactive spots on

autoradiograms and rescreened with probe PCR 7 and riboprobe 1. The rescreen with PCR 7 probe was performed using conditions similar to those used in the initial screen. The rescreen with riboprobe 1 was performed as follows: the filters were prehybridized in 6XSSC, 1% SDS and hybridized at 62°C for 18 h in 0.25 M $NaPO_4$, (pH 7.5), 0.25 M NaCl, 0.001 M EDTA, 15% formamide , 7% SDS and riboprobe at $1X10^6$ cpm/ml. The filters were washed in 6XSSC, 1% SDS for 30 min at 62°C followed by 1XSSC, 1% SDS for 30 min at 62°C. DNA from positive clones was digested with restriction endonucleases Bam HI, SphI or SstI and the resulting fragments were subcloned into pUC119 and subsequently sequenced.

Using probe PCR 7, a clone was obtained that included exons encoding amino acids 40 to 176 and this clone is deposited at the ATCC (deposit #40681). To obtain clones for additional SCF exons, the human genomic library was screened with riboprobe 2 and oligonucleotide probe 235-29. The library was screened in a manner similar to that done previously with the following exceptions: the hybridization with probe 235-29 was done at 37°C and the washes for this hybridization were for 1 h at 37°C and 1 h at 44°C. Positive clones were rescreened with riboprobe 2, riboprobe 3 and oligonucleotide probes 235-29 and 236-31. Riboprobes 2 and 3 were made using a protocol similar to that used to produce riboprobe 1, with the following exceptions: (a) the recombinant pGEM3:hSCF plasmid DNA was linearized with restriction endonuclease PvuII (riboprobe 2) or PstI (riboprobe 3) and (b) the SP6 RNA polymerase (Promega) was used to synthesize riboprobe 3.

Figure 15A shows the strategy used to sequence human genomic DNA. In this figure, the line drawing at the top represents the region of human genomic DNA

encoding SCF. The gaps in the line indicate regions that have not been sequenced. The large boxes represent exons for coding regions of the SCF gene with the corresponding encoded amino acids indicated above each box. The sequence of the human SCF gene is shown in Figure 15B. The sequence of human SCF cDNA obtained PCR techniques is shown in Figure 15C.

F. Sequence of the Human SCF cDNA 5' Region

Sequencing of products from PCRs primed by two gene-specific primers reveals the sequence of the region bounded by the 3' ends of the two primers. One-sided PCRs, as indicated in Example 3A, can yield the sequence of flanking regions. One-sided PCR was used to extend the sequence of the 5'-untranslated region of human SCF cDNA.

First strand cDNA was prepared from poly A+ RNA from the human bladder carcinoma cell line 5637 (ATCC HTB 9) using oligonucleotide 228-28 (Figure 12C) as primer, as described in Example 3D. Tailing of this cDNA with dG residues, followed by one-sided PCR amplification using primers containing $(dC)_n$ sequences in combination with SCF-specific primers, failed to yield cDNA fragments extending upstream (5') of the known sequence.

A small amount of sequence information was obtained from PCR amplification of products of second strand synthesis primed by oligonucleotide 228-28. The untailed 5637 first strand cDNA described above (about 50 ng) and 2 pmol of 228-28 were incubated with Klenow polymerase and 0.5 mM each of dATP, dCTP, dGTP and dTTP at 10-12°C for 30 minutes in 10 uL of 1xNick-translation buffer [Maniatis et al., Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory (1982)]. Amplification of the resulting cDNA by sequential one-

sided PCRs with primer 228-29 in combination with nested SCF primers (in order of use: 235-30, 233-14, 236-31 and finally 235-29) yielded complex product mixtures which appeared as smears on agarose gels. Significant enrichment of SCF-related cDNA fragments was indicated by the increasing intensity of the specific product band observed when comparable volumes of the successive one-sided PCR products were amplified with two SCF primers (227-29 and 235-29, for example, yielding a product of about 150 bp). Attempts to select for a particular size range of products by punching out portions of the agarose gel smears and reamplifying by PCR in most cases failed to yield a well-defined band which contained SCF-related sequences.

One reaction, PCR 16.17, which contained only the 235-29 primer, gave rise to a band which apparently arose from priming by 235-29 at an unknown site 5' of the coding region in addition to the expected site, as shown by mapping with the restriction enzymes PvuII and PstI and PCR analysis with nested primers. This product was gel-purified and reamplified with primer 235-29, and sequencing was attempted by the Sanger dideoxy method using ^{32}P-labelled primer 228-30. The resulting sequence was the basis for the design of oligonucleotide 254-9 (Figure 12B). When this 3' directed primer was used in subsequent PCRs in combination with 5' directed SCF primers, bands of the expected size were obtained. Direct Sanger sequencing of such PCR products yielded nucleotides 180 through 204 of a human SCF cDNA sequence, Figure 15C.

In order to obtain more sequence at the 5' end of the hSCF cDNA, first strand cDNA was prepared from 5637 poly A$^+$ RNA (about 300 ng) using an SCF-specific primer (2 pmol of 233-14) in a 16 uL reaction containing 0.2 U MMLV reverse transcriptase (purchased from BRL) and 500 uM each dNTP. After standard phenol-chloroform

and chloroform extractions and ethanol precipitation (from 1 M ammonium acetate) steps, the nucleic acids were resuspended in 20 uL of water, placed in a boiling water bath for 5 minutes, then cooled and tailed with terminal transferase in the presence of 8 uM dATP in a $CoCl_2$-containing buffer [Deng and Wu, Methods in Enzymology, 100, pp. 96-103]. The product, $(dA)_n$-tailed first-strand cDNA was purified by phenol-chloroform extraction and ethanol precipitation and resuspended in 20 uL of 10mM tris, pH 8.0, and 1mM EDTA.

Enrichment and amplification of human SCF-related cDNA 5' end fragments from about 20 ng of the $(dA)_n$-tailed 5637 cDNA was performed as follows: an initial 26 cycles of one-sided PCR were performed in the presence of SCF-specific primer 236-31 and a primer or primer mixture containing $(dT)_n$ sequences at or near the 3' end, for instance primer 221-12 or a mixture of primers 220-3, 220-7, and 220-11 (Figure 12C). The products (1 µl) of these PCRs were then amplified in a second set of PCRs containing primers 221-12 and 235-29. A major product band of approximately 370 bp was observed in each case upon agarose gel analysis. A gel plug containing part of this band was punched out of the gel with the tip of a Pasteur pipette and transferred to a small microfuge tube. 10 uL of water was added and the plug was melted in an 84°C heating block. A PCR containing primers 221-12 and 235-29 (8 pmol each) in 40 uL was inoculated with 2 uL of the melted, diluted gel plug. After 15 cycles, a slightly diffuse band of approximately 370 bp was visible upon agarose gel analysis. Asymmetric PCRs were performed to generate top and bottom strand sequencing templates: for each reaction, 4 uL of PCR reaction product and 40 pmol of either primer 221-12 or primer 235-29 in a total reaction volume of 100 uL were subjected to 25 cycles of PCR (1 minute, 95°C; 30 seconds, 55°C;

40 seconds, 72°C). Direct sequencing of the 221-12 primed PCR product mixtures (after the standard extractions and ethanol precipitation) with ^{32}P-labelled primer 262-13 (Figure 12B) yielded the 5' sequence from nucleotide 1 to 179 (Figure 15C).

G. Amplification and Sequencing of Human Genomic DNA at the Site of the First Coding Exon of the Stem Cell Factor

Screening of a human genomic library with SCF oligonucleotide probes failed to reveal any clones containing the known portion of the first coding exon. An attempt was then initiated to use a one-sided PCR technique to amplify and clone genomic sequences surrounding this exon.

Primer extension of heat-denatured human placental DNA (purchased from Sigma) was performed with DNA polymerase I (Klenow enzyme, large fragment; Boehringer-Mannheim) using a non-SCF primer such as 228-28 or 221-11 under non-stringent (low temperature) conditions, such as 12°C, to favor priming at a very large number of different sites. Each reaction was then diluted five-fold into TaqI DNA polymerase buffer containing TaqI polymerase and 100 uM of each dNTP, and elongation of DNA strands was allowed to proceed at 72°C for 10 minutes. The product was then enriched for stem cell factor first exon sequences by PCR in the presence of an SCF first exon oligonucleotide (such as 254-9) and the appropriate non-SCF primer (228-29 or 221-11). Agarose gel electrophoresis revealed that most of the products were short (less than 300 bp). To enrich for longer species, the portion of each agarose gel lane corresponding to length greater than 300 bp was cut out and electrophoretically eluted. After ethanol precipitation and resuspension in water, the gel

purified PCR products were cloned into a derivative of pGEM4 containing an SfiI site as a HindIII to SfiI fragment.

Colonies were screened with a ^{32}P-labelled SCF first exon oligonucleotide. Several positive colonies were identified and the sequences of the inserts were obtained by the Sanger method. The resulting sequence, which extends downstream from the first exon through a consensus exon-intron boundary into the neighboring intron, is shown in Figure 15B.

H. Amplification and Sequencing of SCF cDNA Coding Regions from Mouse, Monkey and Dog

First strand cDNA was prepared from total RNA or poly A$^+$ RNA from monkey liver (purchased from Clontech) and from the cell lines NIH-3T3 (mouse, ATCC CRL 1658), and D17 (dog, ATCC CCL 183). The primer used in first strand cDNA synthesis was either the nonspecific primer 228-28 or an SCF primer (227-30, 237-19, 237-20, 230-25 or 241-6). PCR amplification with primer 227-29 and one of the primers 227-30, 237-19 or 237-20 yielded a fragment of the expected size which was sequenced either directly or after cloning into V19.8 or a pGEM vector.

Additional sequences near the 5' end of the SCF cDNAs were obtained from PCR amplifications utilizing an SCF-specific primer in combination with either 254-9 or 228-29. Additional sequences at the 3' end of the SCF coding regions were obtained after PCR amplification of 230-25 primed cDNA (in the case of mouse) or 241-6 primed cDNA (in the case of monkey) with either 230-25 or 241-6, as appropriate, and a 3' directed SCF primer. No SCF PCR product bands were obtained in similar attempts to amplify D17 cDNA. The nonspecific primer 228-28 was used to prime first strand

synthesis from D17 total RNA, and the resulting complex product mixture was enriched for SCF-related sequences by PCR with 3' directed SCF primers such as 227-29 or 225-31 in combination with 228-29. The product mixture was cut with SfiI and cloned into a derivative of pGEM4 (Promega, Madison, Wisconsin) containing an SfiI site as an SfiI to blunt end fragment. The resulting heterogeneous library was screened with radiolabelled 237-20, and several positive clones were sequenced, yielding dog SCF 3' end sequences. The aligned amino acid sequences of human (Figure 42), monkey, dog, mouse and rat SCF mature proteins are shown in Figure 16.

The known SCF amino acid sequences are highly homologous throughout much of their length. Identical consensus signal peptide sequences are present in the coding regions of all five species. The amino acid expected to be at the amino terminus of the mature protein by analogy with the rat SCF is designated by the numeral 1 in this figure. The dog cDNA sequence contains an ambiguity which results in a valine/leucine ambiguity in the amino acid sequence at codon 129. The human, monkey, rat and mouse amino acid sequences co-align without any insertions or deletions. The dog sequence has a single extra residue at position 130 as compared to the other species. Human and monkey differ at only one position, a conservative replacement of valine (human) by alanine (monkey) at position 130. The predicted SCF sequence immediately before and after the putative processing site near residue 164 is highly conserved between species.

EXAMPLE 4

Expression of Recombinant Rat SCF in COS-1 Cells

For transient expression in COS-1 cells (ATCC CRL 1650), vector V19.8 (Example 3C) containing the rat

SCF^{1-162} and SCF^{1-193} genes was transfected into duplicate 60 mm plates [Wigler et al., Cell, 14, 725-731 (1978)]. The plasmid V19.8 SCF is shown in Figure 17. As a control, the vector without insert was also transfected. Tissue culture supernatants were harvested at various time points post-transfection and assayed for biological activity. Table 4 summarizes the HPP-CFC bioassay results and Table 5 summarizes the MC/9 ^3H-thymidine uptake data from typical transfection experiments. Bioassay results of supernatants from COS-1 cells transfected with the following plasmids are shown in Tables 4 and 5: a C-terminally-truncated form of rat SCF with the C-terminus at amino acid position 162 (V19.8 rat SCF^{1-162}), SCF^{1-162} containing a glutamic acid at position 81 [V19.8 rat SCF^{1-162} (Glu81)], and SCF^{1-162} containing an alanine at position 19 [V19.8 rat SCF^{1-162} (Ala19)]. The amino acid substitutions were the product of PCR reactions performed in the amplification of rat SCF^{1-162} as indicated in Example 3. Individual clones of V19.8 rat SCF^{1-162} were sequenced and two clones were found to have amino acid substitutions. As can be seen in Tables 4 and 5, the recombinant rat SCF is active in the bioassays used to purify natural mammalian SCF in Example 1.

Table 4

HPP-CFC Assay of COS-1 Supernatants from Cells Transfected with Rat SCF DNA

Sample	Volume of CM Assayed (µl)	Colony #/200,000 cells
V19.8 (no insert)	100	0
	50	0
	25	0
	12	0
V19.8 rat SCF^{1-162}	100	>50
	50	>50
	25	>50
	12	>50
	6	30
	3	8
V19.8 rat SCF^{1-162} (Glu81)	100	26
	50	10
	25	2
	12	0
V19.8 rat SCF^{1-162} (Ala19)	100	41
	50	18
	25	5
	12	0
	6	0
	3	0

Table 5
MC/9 ^{3}H-Thymidine Uptake Assay of COS-1
Supernatants from Cells Transfected with Rat SCF DNA

Sample	Volume of CM Assayed (ul)	cpm
v19.8(no insert)	25	1,936
	12	2,252
	6	2,182
	3	1,682
v19.8 SCF^{1-162}	25	11,648
	12	11,322
	6	11,482
	3	9,638
v19.8 SCF^{1-162}(Glu81)	25	6,220
	12	5,384
	6	3,692
	3	1,980
v19.8 SCF^{1-162}(Ala19)	25	8,396
	12	6,646
	6	4,566
	3	3,182

Recombinant rat SCF, and other factors, were tested individually in a human CFU-GM [Broxmeyer et al., supra (1977)] assay which measures the proliferation of normal bone marrow cells and the data are shown in Table 6. Results for COS-1 supernatants from cultures 4 days after transfection with V19.8 SCF^{1-162} in combination with other factors are also shown in Table 6. Colony numbers are the average of triplicate cultures.

The recombinant rat SCF has primarily a synergistic activity on normal human bone marrow in the CFU-GM assay. In the experiment in Table 6, SCF synergized with human GM-CSF, human IL-3, and human CSF-1. In other assays, synergy was observed with G-CSF also. There was some proliferation of human bone marrow after 14 days with rat SCF; however, the clusters were composed of <40 cells. Similar results were obtained with natural mammalian-derived SCF.

Table 6

Human CFU-GM Assay of COS-1 Supernatants from Cells Transfected with Rat SCF DNA

Sample	Colony #/100,000 cells (±SEM)
Saline	0
GM-CSF	7 ± 1
G-CSF	24 ± 1
IL-3	5 ± 1
CSF-1	0
SCF^{1-162}	0
GM-CSF + SCF^{1-162}	29 ± 6
G-CSF + SCF^{1-162}	20 ± 1
IL-3 + SCF^{1-162}	11 ± 1
CSF-1 + SCF^{1-162}	4 ± 0

EXAMPLE 5

Expression of Recombinant SCF in Chinese Hamster Ovary Cells

This example relates to a stable mammalian expression system for secretion of SCF from CHO cells (ATCC CCL 61 selected for DHFR-).

A. Recombinant Rat SCF

The expression vector used for SCF production was V19.8 (Figure 17). The selectable marker used to establish stable transformants was the gene for dihydrofolate reductase in the plasmid pDSVE.1. Plasmid pDSVE.1 (Figure 18) is a derivative of pDSVE constructed by digestion of pDSVE by the restriction enzyme SalI and ligation to an oligonucleotide fragment consisting of the two oligonucleotides

5'TCGAC CCGGA TCCCC 3'

3' G GGCCT AGGGG AGCT 5'.

Vector pDSVE is described in commonly owned U.S. Ser. Nos. 025,344 and 152,045 hereby incorporated by reference. The vector portion of V19.8 and pDSVE.1 contain long stretches of homology including a bacterial ColE1 origin of replication and ampicillin resistance gene and the SV40 origin of replication. This overlap may contribute to homologous recombination during the transformation process, thereby facilitating co-transformation.

Calcium phosphate co-precipitates of V19.8 SCF constructs and pDSVE.1 were made in the presence or absence of 10 µg of carrier mouse DNA using 1.0 or 0.1 µg of pDSVE.1 which had been linearized with the restriction endonuclease PvuI and 10 µg of V19.8 SCF as described [Wigler et al., supra (1978)]. Colonies were selected based upon expression of the DHFR gene from

pDSVE.1. Colonies capable of growth in the absence of added hypoxanthine and thymidine were picked using cloning cylinders and expanded as independent cell lines. Cell supernatants from individual cell lines were tested in an MC/9 ^{3}H-thymidine uptake assay. Results from a typical experiment are presented in Table 7.

Table 7

MC/9 ^{3}H-Thymidine Uptake Assay of Stable CHO Cell Supernatants From Cells Transfected With Rat SCF DNA

Transfected DNA	Volume of Conditioned Medium Assayed	cpm
V19.8 SCF$^{1-162}$	25	33,926
	12	34,973
	6	30,657
	3	14,714
	1.5	7,160
None	25	694
	12	1,082
	6	880
	3	672
	1	1,354

B. Recombinant Human SCF

Expression of SCF in CHO cells was also achieved using the expression vector pDSVRα2 which is described in commonly owned Ser. No. 501,904 filed March 29, 1990, hereby incorporated by reference. This vector includes a gene for the selection and amplification of clones based on expression of the DHFR gene. The clone pDSRα2 SCF was generated by a two step process. The V19.8 SCF was digested with the restriction enzyme BamHI and the SCF insert was ligated into the BamHI site of pGEM3.

DNA from pGEM3 SCF was digested with HindIII and SalI and ligated into pDSRα2 digested with HindIII and SalI. The same process was repeated for human genes encoding a COOH-terminus at the amino acid positions 162, 164 and 183 of the sequence shown in Figure 15C and position 248 of the sequences shown in Figure 42. Established cell lines were challenged with methotrexate [Shimke, in Methods in Enzymology, 151 85-104 (1987)] at 10 nM to increase expression levels of the DHFR gene and the adjacent SCF gene. Expression levels of recombinant human SCF were assayed by radioimmune assay, as in Example 7, and/or induction of colony formation in vitro using human peripheral blood leucocytes. This assay is performed as described in Example 9 (Table 12) except that peripheral blood is used instead of bone marrow and the incubation is performed at 20% 0_2, 5% CO_2, and 75% N_2 in the presence of human EPO (10 U/ml). Results from typical experiments are shown in Table 8. The CHO clone expressing human SCF^{1-164} has been deposited on September 25, 1990 with ATCC (CRL 10557) and designated Hu164SCF17.

Table 8

hPBL Colony Assay of Conditioned Media From Stable CHO Cell Lines Transfected With Human SCF DNA

Transfected DNA	Media assayed(µl)	Number of Colonies/10^5
pDSRα2 $hSCF^{1-164}$	50	53
	25	45
	12.5	27
	6.25	13
pDSRα2 $hSCF^{1-162}$	10	43
	5	44
	2.5	31
	1.25	17
	0.625	21
None (CHO control)	50	4

EXAMPLE 6

Expression of Recombinant SCF in E. coli

A. Recombinant Rat SCF

This example relates to expression in E. coli of SCF polypeptides by means of a DNA sequence encoding [Met^{-1}] rat SCF^{1-193} (Figure 14C). Although any suitable vector may be employed for protein expression using this DNA, the plasmid chosen was pCFM1156 (Figure 19). This plasmid can be readily constructed from pCFM 836 (see U.S. Patent No. 4,710,473 hereby incorporated by reference) by destroying the two endogenous NdeI restriction sites by end-filling with T4 polymerase enzyme followed by blunt end ligation and

substituting the small DNA sequence between the unique ClaI and KpnI restriction sites with the small oligonucleotide shown below.

```
5'  CGATTTGATTCTAGAAGGAGGAATAACATATGGTTAACGCGTTGGAATTCGGTAC 3'
3'    TAAACTAAGATCTTCCTCCTTATTGTATACCAATTGCGCAACCTTAAGC    5'
```

Control of protein expression in the pCFM1156 plasmid is by means of a synthetic lambda P_L promoter which is itself under the control of a temperature sensitive lambda CI857 repressor gene [such as is provided in E. coli strains FM5 (ATCC deposit #53911) or K12ΔHtrp]. The pCFM1156 vector is constructed so as to have a DNA sequence containing an optimized ribosome binding site and initiation codon immediately 3' of the synthetic PL promoter. A unique NdeI restriction site, which contains the ATG initiation codon, precedes a multi-restriction site cloning cluster followed by a lambda t-oop transcription stop sequence.

Plasmid V19.8 SCF^{1-193} containing the rat SCF^{1-193} gene cloned from PCR amplified cDNA (Figure 14C) as described in Example 3 was digested with BglII and SstII and a 603 bp DNA fragment isolated. In order to provide a Met initiation codon and restore the codons for the first three amino acid residues (Gln, Glu, and Ile) of the rat SCF polypeptide, a synthetic oligonucleotide linker

```
5'  TATGCAGGA    3'
3'    ACGTCCTCTAG  5'
```

with NdeI and BglII sticky ends was made. The small oligonucleotide and rat SCF^{1-193} gene fragment were inserted by ligation into pCFM1156 at the unique NdeI and SstII sites in the plasmid shown in Figure 19. The product of this reaction is an expression plasmid, pCFM1156 rat SCF^{1-193}.

The pCFM1156 rat SCF^{1-193} plasmid was transformed into competent FM5 E. coli host cells. Selection for plasmid-containing cells was on the basis of the antibiotic (kanamycin) resistance marker gene carried on the pCFM1156 vector. Plasmid DNA was isolated from cultured cells and the DNA sequence of the synthetic oligonucleotide and its junction to the rat SCF gene confirmed by DNA sequencing.

To construct the plasmid pCFM1156 rat SCF^{1-162} encoding the [Met^{-1}] rat SCF^{1-162} polypeptide, an EcoRI to SstII restriction fragment was isolated from V19.8 rat SCF^{1-162} and inserted by ligation into the plasmid pCFM rat SCF^{1-193} at the unique EcoRI and SstII restriction sites thereby replacing the coding region for the carboxyl terminus of the rat SCF gene.

To construct the plasmids pCFM1156 rat SCF^{1-164} and pCFM1156 rat SCF^{1-165} encoding the [Met^{-1}] rat SCF^{1-164} and [Met^{-1}] rat SCF^{1-165} polypetides, respectively, EcoRI to SstII restriction fragments were isolated from PCR amplified DNA encoding the 3' end of the SCF gene and designed to introduce site directed changes in the DNA in the region encoding the carboxyl terminus of the SCF gene. The DNA amplifications were performed using the oligonucleotide primers 227-29 and 237-19 in the construction of pCFM1156 rat SCF^{1-164} and 227-29 and 237-20 in the construction of pCFM1156 rat SCF^{1-165}.

B. Recombinant Human SCF

This example relates to the expression in E. coli of human SCF polypeptide by means of a DNA sequence encoding [Met^{-1}] human SCF^{1-164} and [Met^{-1}] human SCF^{1-183} (Figure 15C). Plasmid V19.8 human SCF^{1-162} containing the human SCF^{1-162} gene was used as template for PCR amplification of the human SCF gene.

Oligonucleotide primers 227-29 and 237-19 were used to generate the PCR DNA which was then digested with PstI and SstII restriction endonucleases. In order to provide a Met initiation codon and restore the codons for the first four amino acid residues (Glu, Gly, Ile, Cys) of the human SCF polypeptide, a synthetic oligonucleotide linker

5' TATGGAAGGTATCTGCA 3'
3' ACCTTCCATAG 5'

with NdeI and PstI sticky ends was made. The small oligo linker and the PCR derived human SCF gene fragment were inserted by ligation into the expression plasmid pCFM1156 (as described previously) at the unique NdeI and SstII sites in the plasmid shown in Figure 19.

The pCFM1156 human SCF^{1-164} plasmid was transformed into competent FM5 E. coli host cells. Selection for plasmid containing cells was on the basis of the antibiotic (kanamycin) resistance marker gene carried on the pCFM1156 vector. Plasmid DNA was isolated from cultured cells and the DNA sequence of the human SCF gene confirmed by DNA sequencing.

To construct the plasmid pCFM1156 human SCF^{1-183} encoding the [Met^{-1}] human SCF^{1-183} (Figure 15C) polypeptide, a EcoRI to HindIII restriction fragment encoding the carboxyl terminus of the human SCF gene was isolated from pGEM human $SCF^{114-183}$ (described below), a SstI to EcoRI restriction fragment encoding the amino terminus of the human SCF gene was isolated from pCFM1156 human SCF^{1-164}, and the larger HindIII to SstI restriction fragment from pCFM1156 was isolated. The three DNA fragments were ligated together to form the pCFM1156 human SCF^{1-183} plasmid which was then tranformed into FM5 E. coli host cells. After colony selection using kanamycin drug resistance, the plasmid

DNA was isolated and the correct DNA sequence confirmed by DNA sequencing. The pGEM human $SCF^{114-183}$ plasmid is a derivative of pGEM3 that contains an EcoRI-SphI fragment that includes nucleotides 609 to 820 of the human SCF cDNA sequence shown in Figure 15C. The EcoRI-SphI insert in this plasmid was isolated from a PCR that used oligonucleotide primers 235-31 and 241-6 (figure 12B) and PCR 22.7 (Figure 13B) as template. The sequence of primer 241-6 was based on the human genomic sequence to the 3' side of the exon containing the codon for amino acid 176.

C. Fermentation of E. coli producing Human SCF^{1-164}

Fermentations for the production of SCF 1-164 were carried out in 16 liter fermentors using an FM5 E. coli K12 host containing the plasmid pCFM 1156 human SCF^{1-164}. Seed stocks of the producing culture were maintained at -80° C in 17% glycerol in Luria broth. For inoculum production, 100 µl of the thawed seed stock was transferred to 500 ml of Luria broth in a 2 L erlenmeyer flask and grown overnight at 30°C on a rotary shaker (250 RPM).

For the production of E. coli cell paste used as starting material for the purification of human SCF^{1-164} outlined in this example, the following fermentation conditions were used.

The inoculum culture was aseptically transferred to a 16 L fermentor containing 8 L of batch medium (see Table 9). The culture was grown in batch mode until the OD-600 of the culture was approximately 3.5. At this time, a sterile feed (Feed 1, Table 10) was introduced into the fermentor using a peristaltic pump to control the feed rate. The feed rate was increased exponentially with time to give a growth rate of 0.15 hr^{-1}. The temperature was controlled at 30°C

during the growth phase. The dissolved oxygen concentration in the fermentor was automatically controlled at 50% saturation using air flow rate, agitation rate, vessel back pressure and oxygen supplementation for control. The pH of the fermentor was automatically controlled at 7.0 using phosphoric acid and ammonium hydroxide. At an OD-600 of approximately 30, the production phase of the fermentation was induced by increasing the fermentor temperature to 42°C. At the same time the addition of Feed 1 was stopped and the addition of Feed 2 (Table 11) was started at a rate of 200 ml/hr. Approximately six hours after the temperature of the fermentor was increased, the fermentor contents were chilled to 15°C. The yield of SCF^{1-164} was approximately 30 mg/OD-L. The cell pellet was then harvested by centrifugation in a Beckman J6-B rotor at 3000 x g for one hour. The harvested cell paste was stored frozen at -70°C.

A preferred method for production of SCF^{1-164} is similar to the method described above except for the following modifications.

1) The addition of Feed 1 is not initiated until the OD-600 of the culture reaches 5-6.

2) The rate of addition of Feed 1 is increased more slowly, resulting in a slower growth rate (approximately 0.08).

3) The culture is induced at OD-600 of 20.

4) Feed 2 is introduced into the fermentor at a rate of 300 mL/hr.

All other operations are similar to the method described above, including the media.

Using this process, yields of SCF^{1-164} approximately 35-40 mg/OD-L at OD=25 have been obtained.

TABLE 9

Composition of Batch Medium

Yeast extract	10[a] g/L
Glucose	5
K_2HPO_4	3.5
KH_2PO_4	4
$M_GSO_4 \cdot 7H_2O$	1
NaCl	0.625
Dow P-2000 antifoam	5 mL/8 L
Vitamin solution[b]	2 mL/L
Trace metals solution[c]	2 mL/L

[a]Unless otherwise noted, all ingredients are listed as g/L.

[b]Trace Metals solution: $FeCl_3 \cdot 6H_2O$, 27 g/L; $ZnCl_2 \cdot 4 H_2O$, 2g/L; $CaCl_2 \cdot 6H_2O$, 2 g/L; $Na_2MoO_4 \cdot 2 H_2O$, 2 g/L, $CuSO_4 \cdot 5 H_2O$, 1.9 g/L; concentrated HCl, 100 ml/L.

[c]Vitamin solution: riboflavin, 0.42 g/l; pantothenic acid, 5.4 g/L; niacin, 6 g/L; pyridoxine, 1.4 g/L; biotin, 0.06 g/L; folic acid, 0.04 g/L.

TABLE 10

Composition of Feed Medium

Yeast extract	50[a]
Glucose	450
$MgSO_4 \cdot 7H_2O$	8.6
Trace metals solution[b]	10 mL/L
Vitamin solution[c]	10 mL/L

[a]Unless otherwise noted, all ingredients are listed as g/L.

[b]Trace Metals solution: $FeCl_3 \cdot 6H_2O$, 27 g/L; $ZnCl_2 \cdot 4 H_2O$, 2g/L; $CaCl_2 \cdot 6H_2O$, 2 g/L; $Na_2MoO_4 \cdot 2 H_2O$, 2 g/L, $CuSO_4 \cdot 5 H_2O$, 1.9 g/L; concentrated HCl, 100 ml/L.

[c]Vitamin solution: riboflavin, 0.42 g/l; pantothenic acid, 5.4 g/L; niacin, 6 g/L; pyridoxine, 1.4 g/L; biotin, 0.06 g/L; folic acid, 0.04 g/L.

TABLE 11

Composition of Feed Medium 2

Tryptone	172[a]
Yeast extract	86
Glucose	258

[a]All ingredients are listed as g/L.

EXAMPLE 7

Immunoassays for Detection of SCF

Radioimmunoassay (RIA) procedures applied for quantitative detection of SCF in samples were conducted according to the following procedures.

An SCF preparation from BRL 3A cells purified as in Example 1 was incubated together with antiserum for two hours at 37°C. After the two hour incubation, the sample tubes were then cooled on ice, ^{125}I-SCF was added, and the tubes were incubated at 4°C for at least 20 h. Each assay tube contained 500 µl of incubation mixture consisting of 50 µl of diluted antisera, ~60,000 cpm of ^{125}I-SCF (3.8×10^7 cpm/µg), 5 µl trasylol and 0-400 µl of SCF standard, with buffer (phosphate buffered saline, 0.1% bovine serum albumin, 0.05% Triton X-100, 0.025% azide) making up the remaining volume. The antiserum was the second test bleed of a rabbit immunized with a 50% pure preparation of natural SCF from BRL 3A conditioned medium. The final antiserum dilution in the assay was 1:2000.

The antibody-bound ^{125}I-SCF was precipitated by the addition of 150 µl Staph A (Calbiochem). After a 1 h incubation at room temperature, the samples were centrifuged and the pellets were washed twice with 0.75 ml 10 mM Tris-HCL pH 8.2, containing 0.15M NaCl,

2 mM EDTA, and 0.05% Triton X-100. The washed pellets were counted in a gamma counter to determine the percent of ^{125}I-SCF bound. Counts bound by tubes lacking serum were subtracted from all final values to correct for nonspecific precipitation. A typical RIA is shown in Figure 20. The percent inhibition of ^{125}I-SCF binding produced by the unlabeled standard is dose dependent (Figure 20A), and, as indicated in Figure 20B, when the immune precipitated pellets are examined by SDS-PAGE and autoradiography, the ^{125}I-SCF protein band is competed. In Figure 20B, lane 1 is ^{125}I-SCF, and lanes 2, 3, 4 and 5 are immune-precipicated ^{125}I-SCF competed with 0, 2, 100, and 200 ng of SCF standard, respectively. As determined by both the decrease in antibody-precipitable cpm observed in the RIA tubes and decrease in the immune-precipitated ^{125}I-SCF protein band (migrating at approximately M_r 31,000) the polyclonal antisera recognizes the SCF standard which was purified as in Example 1.

Western procedures were also applied to detect recombinant SCF expressed in E. coli, COS-1, and CHO cells. Partially purified E. coli expressed rat SCF^{1-193} (Example 10), COS-1 cell expressed rat SCF^{1-162} and SCF^{1-193} as well as human SCF^{1-162} (Examples 4 and 9), and CHO cell expressed rat SCF^{1-162} (Example 5), were subjected to SDS-PAGE. Following electrophoresis, the protein bands were transferred to 0.2 µm nitrocellulose using a Bio-Rad Transblot apparatus at 60V for 5 h. The nitrocellulose filters were blocked for 4 h in PBS, pH 7.6, containing 10% goat serum followed by a 14 h room temperature incubation with a 1:200 dilution of either rabbit preimmune or immune serum (immunization described above). The antibody-antiserum complexes were visualized using horseradish peroxidase-conjugated goat anti-rabbit IgG reagents (Vector laboratories) and 4-chloro-1-napthol color development reagent.

Examples of two Western analyses are presented in Figures 21 and 22. In Figure 21, lanes 3 and 5 are 200 µl of COS-1 cell produced human SCF^{1-162}; lanes 1 and 7 are 200 µl of COS-1 cell produced human EPO (COS-1 cells transfected with V19.8 EPO); and lane 8 is prestained molecular weight markers. Lanes 1-4 were incubated with pre-immune serum and lanes 5-8 were incubated with immune serum. The immune serum specifically recognizes a diffuse band with an apparent M_r of 30,000 daltons from COS-1 cells producing human SCF^{1-162} but not from COS-1 cells producing human EPO.

In the Western shown in Figure 22, lanes 1 and 7 are 1 µg of a partially purified preparation of rat SCF^{1-193} produced in E. coli; lanes 2 and 8 are wheat germ agglutinin-agarose purified COS-1 cell produced rat SCF^{1-193}; lanes 4 and 9 are wheat germ agglutinin-agarose purified COS-1 cell produced rat SCF^{1-162}; lanes 5 and 10 are wheat germ agglutinin-agarose purified CHO cell produced rat SCF^{1-162}; and lane 6 is prestained molecular weight markers. Lanes 1-5 and lanes 6-10 were incubated with rabbit preimmune and immune serum, respectively. The E. coli produced rat SCF^{1-193} (lanes 1 and 7) migrates with an apparent M_r of ~24,000 daltons while the COS-1 cell produced rat SCF^{1-193} (lanes 2 and 8) migrates with an apparent M_r of 24-36,000 daltons. This difference in molecular weights is expected since mammalian cells, but not bacteria, are capable of glycosylation. Transfection of the sequence encoding rat SCF^{1-162} into COS-1 (lanes 4 and 9), or CHO cells (lanes 5 and 10), results in expression of SCF with a lower average molecular weight than that produced by transfection with SCF^{1-193} (lanes 2 and 8).

The expression products of rat SCF^{1-162} from COS-1 and CHO cells are a series of bands ranging in apparent M_r between 24-36,000 daltons. The heterogeneity of the expressed SCF is likely due to

carbohydrate variants, where the SCF polypeptide is glycosylated to different extents.

In summary, Western analyses indicate that immune serum from rabbits immunized with natural mammalian SCF recognize recombinant SCF produced in E. coli, COS-1 and CHO cells but fail to recognize any bands in a control sample consisting of COS-1 cell produced EPO. In further support of the specificity of the SCF antiserum, preimmune serum from the same rabbit failed to react with any of the rat or human SCF expression products.

EXAMPLE 8

In Vivo Activity of Recombinant SCF

A. Rat SCF in Bone Marrow Transplanation

COS-1 cells were transfected with V19.8 SCF^{1-162} in a large scale experiment (T175 cm^2 flasks instead of 60 mm dishes) as described in Example 4. Approximately 270 ml of supernatant was harvested. This supernatant was chromatographed on wheat germ agglutinin-agarose and S-Sepharose essentially as described in Example 1. The recombinant SCF was evaluated in a bone marrow transplantation model based on murine W/W^V genetics. The W/W^V mouse has a stem cell defect which among other features results in a macrocytic anemia (large red cells) and allows for the transplantation of bone marrow from normal animals without the need for irradiation of the recipient animals [Russel, et al., Science, 144, 844-846 (1964)]. The normal donor stem cells outgrow the defective recipient cells after transplantation.

In the following example, each group contained six age matched mice. Bone marrow was harvested from normal donor mice and transplanted into W/W^V mice. The

blood profile of the recipient animals is followed at different times post transplantation and engraftment of the donor marrow is determined by the shift of the peripheral blood cells from recipient to donor phenotype. The conversion from recipient to donor phenotype is detected by monitoring the forward scatter profile (FASCAN, Becton Dickenson) of the red blood cells. The profile for each transplanted animal was compared to that for both donor and recipient un-transplanted control animals at each time point. The comparison was made utilizing a computer program based on Kolmogorov-Smirnov statistics for the analysis of histograms from flow systems [Young, J. Histochem. and Cytochem., 25, 935-941 (1977)]. An independent qualitative indicator of engraftment is the hemoglobin type detected by hemoglobin electrophoresis of the recipient blood [Wong, et al., Mol. and Cell. Biol., 9, 798-808 (1989)] and agrees well with the goodness of fit determination from Kolmogorov-Smirnov statistics.

Approximately 3 x 10^5 cells were transplanted without SCF treatment (control group in Figure 23) from C56BL/6J donors into W/W^v recipients. A second group received 3 x 10^5 donor cells which had been treated with SCF (600 U/ml) at 37°C for 20 min and injected together (pre-treated group in Figure 23). (One unit of SCF is defined as the amount which results in half-maximal stimulation in the MC/9 bioassay). In a third group, the recipient mice were injected sub-cutaneously (sub-Q) with approximately 400 U SCF/day for 3 days after transplantation of 3 x 10^5 donor cells (Sub-Q inject group in Figure 23). As indicated in Figure 23, in both SCF-treated groups the donor marrow is engrafted faster than in the untreated control group. By 29 days post-transplantation, the SCF pre-treated group had converted to donor phenotype. This Example illustrates the usefulness of SCF therapy in bone marrow transplantation.

B. In vivo activity of Rat SCF in Steel Mice

Mutations at the *Sl* locus cause deficiencies in hematopoietic cells, pigment cells, and germ cells. The hematopoietic defect is manifest as reduced numbers of red blood cells [Russell, In:Al Gordon, Regulation of Hematopoiesis, Vol. I, 649-675 Appleton-Century-Crafts, New York (1970)], neutrophils [Ruscetti, Proc. Soc. Exp. Biol. Med., 152, 398 (1976)], monocytes [Shibata, J. Immunol. 135, 3905 (1985)], megakaryocytes [Ebbe, Exp. Hematol., 6, 201 (1978)], natural killer cells [(Clark, Immunogenetics, 12, 601 (1981)], and mast cells [Hayashi, Dev. Biol., 109, 234 (1985)]. Steel mice are poor recipients of a bone marrow transplant due to a reduced ability to support stem cells [Bannerman, Prog. Hematol., 8, 131 (1973)]. The gene encoding SCF is deleted in Steel (*Sl/Sl*) mice.

Steel mice provide a sensitive in vivo model for SCF activity. Different recombinant SCF proteins were tested in Steel-Dickie (Sl/Sl^d) mice for varying lengths of time. Six to ten week old Steel mice (WCB6F1-Sl/Sl^d) were purchased from Jackson Labs, Bar Harbor, ME. Peripheral blood was monitored by a SYSMEX F-800 microcell counter (Baxter, Irvine, CA) for red cells, hemoglobin, and platelets. For enumeration of peripheral white blood cell (WBC) numbers, a Coulter Channelyzer 256 (Coulter Electronics, Marietta, GA) was used.

In the experiment in Figure 24, Steel-Dickie mice were treated with E. coli derived SCF 1-164, purified as in Example 10, at a dose of 100 µg/kg/day for 30 days, then at a dose of 30 µg/kg/day for an additional 20 days. The protein was formulated in injectable saline (Abbott Labs, North Chicago, IL) +0.1% fetal bovine serum. The injections were

performed daily, subcutaneously. The peripheral blood was monitored via tail bleeds of ~50 µl at the indicated times in Figure 24. The blood was collected into 3% EDTA coated syringes and dispensed into powdered EDTA microfuge tubes (Brinkmann, Westbury, NY). There is a significant correction of the macrocytic anemia in the treated animals relative to the control animals. Upon cessation of treatment, the treated animals return to the initial state of macrocytic anemia.

In the experiment shown in Figure 25 and 26, Steel-Dickie mice were treated with different recombinant forms of SCF as described above, but at a dose of 100 µg/kg/day for 20 days. Two forms of *E. coli* derived rat SCF, SCF^{1-164} and SCF^{1-193}, were produced as described in Example 10. In addition, *E. coli* SCF^{1-164}, modified by the addition of polyethylene glycol (SCF^{1-164} PEG25) as in Example 12, was also tested. CHO derived SCF^{1-162} produced as in Example 5 and purified as in Example 11, was also tested. The animals were bled by cardiac puncture with 3% EDTA coated syringes and dispensed into EDTA powdered tubes. The peripheral blood profiles after 20 days of treatment are shown in Figure 25 for white blood cells (WBC) and Figure 26 for platelets. The WBC differentials for the SCF^{1-164} PEG25 group are shown in Figure 27. There are absolute increases in neutrophils, monocytes, lymphocytes, and platelets. The most dramatic effect is seen with SCF^{1-164} PEG 25.

An independent measurement of lymphocyte subsets was also performed and the data is shown in Figure 28. The murine equivalent of human CD4, or marker of T helper cells, is L3T4 [Dialynas, *J. Immunol.*, 131, 2445 (1983)]. LyT-2 is a murine antigen on cytotoxic T cells [Ledbetter, *J. Exp. Med.*, 153, 1503 (1981)]. Monoclonal antibodies against these

antigens were used to evaluate T cell subsets in the treated animals.

Whole blood was stained for T lymphocyte subsets as follows. Two hundred microliters of whole blood was drawn from individual animals into EDTA treated tubes. Each sample of blood was lysed with sterile deionized water for 60 seconds and then made isotonic with 10X Dulbecco's Phosphate Buffered Saline (PBS) (Gibco, Grand Island, NY). This lysed blood was washed 2 times with 1X PBS (Gibco, Grand Island, NY) supplemented with 0.1% Fetal Bovine Serum (Flow Laboratory, McLean, VA) and 0.1% sodium azide. Each sample of blood was deposited into round bottom 96 well cluster dishes and centrifuged. The cell pellet (containing 2-10 x 10^5 cells) was resuspended with 20 microliters of Rat anti-Mouse L3T4 conjugated with phycoerythrin (PE) (Becton Dickinson, Mountain View, CA) and 20 microliters of Rat anti-Mouse Lyt-2 conjugated with Fluorescein Isothiocyanate incubated on ice (4°C) for 30 minutes (Becton Dickinson). Following incubation the cells were washed 2 times in 1X PBS supplemented as indicated aboved. Each sample of blood was then analyzed on a FACScan cell analysis system (Becton Dickinson, Mountain View, CA). This system was standardized using standard autocompensation procedures and Calibrite Beads (Becton Dickinson, Mountain View, CA). These data indicated an absolute increase in both helper T cell populations as well as cytotoxic T cell numbers.

C. In vivo activity of SCF in primates

Human SCF 1-164 expressed in E. coli (Example 6B) and purified to homogeneity as in Example 10, was tested for in vivo biological activity in normal primates. Adult male baboons (Papio sp.)

were studied in three groups: untreated, n=3; SCF 100 ug/kg/day, n=6; and SCF 30 ug/kg/day, n=6. The treated animals received single daily subcutaneous injections of SCF. Blood specimens were obtained from the animals under ketamine restraint. Specimens for complete blood count, reticulocyte count, and platelet count were obtained on days 1, 6, 11, 15, 20 and 25 of treatment.

All animals survived the protocol and had no adverse reactions to SCF therapy. The white blood cell count increased in the 100 ug/kg treated animals as depicted in Figure 29. The differential count, obtained manually from Wright Giemsa stained peripheral blood smears, is also indicated in Figure 29. There was an absolute increase in neutrophils, lymphocytes, and monocytes. As indicated in Figure 30 there was also an increase at the 100 ug/kg dose in the hemtocrits as well as platelets.

Human SCF ($hSCF^{1-164}$ modified by the addition of polyethylene glycol as in Example 12) was also tested in normal baboons, at a dose of 200 µg/kg-day, administered by continuous intravenous infusion and compared to the unmodified protein. The animals started SCF at day 0 and were treated for 28 days. The results for the peripheral WBC are given in the following table. The PEG modified SCF elicited an earlier rise in peripheral WBC than the unmodified SCF.

Treatment with 200 µg/kg-day hSCF$^{1-164}$:

Animal # M88320		Animal # M88129	
DAY	WBC	DAY	WBC
0	5800	0	6800
+7	10700	+7	7400
+14	12600	+14	20900
+16	22000	+21	18400
+22	31100	+23	24900
+24	28100	+29	13000
+29	9600	+30	23000
+36	6600	+37	12100
+43	5600	+44	10700
		+51	7800

Treatment with 200 µg/kg-day PEG-hSCF$^{1-164}$:

Animal # M88350		Animal # M89116	
DAY	WBC	DAY	WBC
-7	12400	-5	7900
-2	11600	0	7400
+4	24700	+6	16400
+7	20400	+9	17100
+11	24700	+13	18700
+14	32600	+16	19400
+18	33600	+20	27800
+21	26400	+23	20700
+25	16600	+27	20200
+28	26900	+29	18600
+32	9200	+33	7600

EXAMPLE 9

In vitro Activity of Recombinant Human SCF

The cDNA of human SCF corresponding to amino acids 1-162 obtained by PCR reactions outlined in Example 3D, was expressed in COS-1 cells as described for the rat SCF in Example 4. COS-1 supernatants were assayed on human bone marrow as well as in the murine HPP-CFC and MC/9 assays. The human protein was not active at the concentrations tested in either murine assay; however, it was active on human bone marrow. The culture conditions of the assay were as follows: human bone marrow from healthy volunteers was centrifuged over Ficoll-Hypaque gradients (Pharmacia) and cultured in 2.1% methyl cellulose, 30% fetal calf serum, 6×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, ISCOVE'S medium (GIBCO), 20 U/ml EPO, and 1×10^5 cells/ml for 14 days in a humidified atmosphere containing 7% O_2, 10% CO_2, and 83% N_2. The colony numbers generated with recombinant human and rat SCF COS-1 supernatants are indicated in Table 12. Only those colonies of 0.2 mm in size or larger are indicated.

Table 12

Growth of Human Bone Marrow Colonies in Response to SCF

Plasmid Transfected	Volume of CM Assayed (µl)	Colony #/100,000 cells ± SD
V19.8 (no insert)	100	0
	50	0
V19.8 human SCF^{1-162}	100	33±7
	50	22±3
V19.8 rat SCF^{1-162}	100	13±1
	50	10

The colonies which grew over the 14 day period are shown in Figure 31A (magnification 12x). The arrow indicates a typical colony. The colonies resembled the murine HPP-CFC colonies in their large size (average 0.5 mm). Due to the presence of EPO, some of the colonies were hemoglobinized. When the colonies were isolated and centrifuged onto glass slides using a Cytospin (Shandon) followed by staining with Wright-Giemsa, the predominant cell type was an undifferentiated cell with a large nucleus:cytoplasm ratio as shown in Figure 31B (magnification 400x). The arrows in Figure 31B point to the following structures: arrow 1, cytoplasm; arrow 2, nucleus; arrow 3, vacuoles. Immature cells as a class are large and the cells become progressively smaller as they mature [Diggs et al., The Morphology of Human Blood Cells, Abbott Labs, 3 (1978)]. The nuclei of early cells of the hemotopoietic maturation sequence are relatively large in relation to the cytoplasm. In addition, the cytoplasm of immature cells stains darker with Wright-Giemsa than does the nucleus. As cells mature, the nucleus stains darker than the cytoplasm. The morphology of the human bone marrow cells resulting from culture with recombinant human SCF is consistent with the conclusion that the target and immediate product of SCF action is a relatively immature hematopoietic progenitor.

Recombinant human SCF was tested in agar colony assays on human bone marrow in combination with other growth factors as described above. The results are shown in Table 13. SCF synergizes with G-CSF, GM-CSF, IL-3, and EPO to increase the proliferation of bone marrow targets for the individual CSFs.

TABLE 13.

Recombinant human SCF Synergy with Other Human Colony Stimulating Factors

	Colony #/10^5 cells (14 Days)
mock	0
hG-CSF	32 ± 3
hG-CSF + hSCF	74 ± 1
hGM-CSF	14 ± 2
hGM-CSF + hSCF	108 ± 5
hIL-3	23 ± 1
hIL-3 + hSCF	108 ± 3
hEPO	10 ± 5
hEPO + IL-3	17 ± 1
hEPO + hSCF	86 ± 10
hSCF	0

Another activity of recombinant human SCF is the ability to cause proliferation in soft agar of the human acute myelogenous leukemia (AML) cell line, KG-1 (ATCC CCL 246). COS-1 supernatants from transfected cells were tested in a KG-1 agar cloning assay [Koeffler et al., Science, 200, 1153-1154 (1978)] essentially as described except cells were plated at 3000/ml. The data from triplicate cultures are given in Table 14.

Table 14

KG-1 Soft Agar Cloning Assay

Plasmid Transfected	Volume Assayed (µl)	Colony #/3000 Cells ± SD
V19.8 (no insert)	25	2±1
V19.8 human SCF^{1-162}	25	14±0
	12	8±0
	6	9±5
	3	6±4
	1.5	6±6
V19.8 rat SCF^{1-162}	25	6±1
human GM-CSF	50 (5 ng/ml)	14±5

EXAMPLE 10

Purification of Recombinant SCF Products Expressed in E. coli

Fermentation of E. coli human SCF^{1-164} was performed according to Example 6C. The harvested cells (912 g wet weight) were suspended in water to a volume of 4.6 L and broken by three passes through a laboratory homogenizer (Gaulin Model 15MR-8TBA) at 8000 psi. A broken cell pellet fraction was obtained by centrifugation (17700 x g, 30 min, 4°C), washed once with water (resuspension and recentrifugation), and finally suspended in water to a volume of 400 ml.

The pellet fraction containing insoluble SCF (estimate of 10-12 g SCF) was added to 3950 ml of an appropriate mixture such that the final concentrations of components in the mixture were 8 M urea (ultrapure grade), 0.1 mM EDTA, 50 mM sodium acetate, pH 6-7; SCF concentration was estimated as 1.5 mg/ml. Incubation was carried out at room temperature for 4 h to solubilize the SCF. Remaining insoluble material was removed by centrifugation (17700 x g, 30 min, room

temperature). For refolding/reoxidation of the solubilized SCF, the supernatant fraction was added slowly, with stirring, to 39.15 L of an appropriate mixture such that the final concentrations of components in the mixture were 2.5 M urea (ultrapure grade), 0.01 mM EDTA, 5 mM sodium acetate, 50 mM Tris-HCl pH 8.5, 1 mM glutathione, 0.02% (wt/vol) sodium azide. SCF concentration was estimated as 150 µg/ml. After 60 h at room temperature [shorter times (e.g. ~20 h) are suitable also], with stirring, the mixture was concentrated two-fold using a Millipore Pellicon ultrafiltration apparatus with three 10,000 molecular weight cutoff polysulfone membrane cassettes (15 ft^2 total area) and then diafiltered against 7 volumes of 20 mM Tris-HCl, pH 8. The temperature during the concentration/ultrafiltration was 4°C, pumping rate was 5 L/min, and filtration rate was 600 ml/min. The final volume of recovered retentate was 26.5 L. By the use of SDS-PAGE carried out both with and without reduction of samples, it is evident that most (>80%) of the pellet fraction SCF is solubilized by the incubation with 8 M urea, and that after the folding/oxidation multiple species (forms) of SCF are present, as visualized by the SDS-PAGE of unreduced samples. The major form, which represents correctly oxidized SCF (see below), migrates with apparent M_r of about 17,000 (unreduced) relative to the molecular weight markers (reduced) described for Figure 9. Other forms include material migrating with apparent M_r of about 18-20,000 (unreduced), thought to represent SCF with incorrect intrachain disulfide bonds; and bands migrating with apparent M_rs in the range of 37,000 (unreduced), or greater, thought to represent various SCF forms having interchain disulfide bonds resulting in SCF polypeptide chains that are covalently linked to form dimers or larger oligomers, respectively. The following fractionation steps result

in removal of remaining E. coli contaminants and of the unwanted SCF forms, such that SCF purified to apparent homogeneity, in biologically active conformation, is obtained.

The pH of the ultrafiltration retentate was adjusted to 4.5 by addition of 375 ml of 10% (vol/vol) acetic acid, leading to the presence of visible precipitated material. After 60 min, at which point much of the precipitated material had settled to the bottom of the vessel, the upper 24 L were decanted and filtered through a Cuno™ 30SP depth filter at 500 ml/min to complete the clarification. The filtrate was then diluted 1.5-fold with water and applied at 4°C to an S-Sepharose Fast Flow (Pharmacia) column (9 x 18.5 cm) equilibrated in 25 mM sodium acetate, pH 4.5. The column was run at a flow rate of 5 L/h, at 4°C. After sample application, the column was washed with five column volumes (~6 L) of column buffer and SCF material, which was bound to the column, was eluted with a gradient of 0 to 0.35 M NaCl in column buffer. Total gradient volume was 20 L and fractions of 200 ml were collected. The elution profile is depicted in Figure 33. Aliquots (10 µl) from fractions collected from the S-Sepharose column were analyzed by SDS-PAGE carried out both with (Figure 32 A) and without (Figure 32 B) reduction of the samples. From such analyses it is apparent that virtually all of the absorbance at 280 nm (Figures 32 and 33) is due to SCF material.

The correctly oxidized form predominates in the major absorbance peak (fractions 22-38, Figure 33). Minor species (forms) which can be visualized in fractions include the incorrectly oxidized material with apparent M_r of 18-20,000 on SDS-PAGE (unreduced), present in the leading shoulder of the main absorbance peak (fractions 10-21, Figure 32 B); and

disulfide-linked dimer material present throughout the absorbance region (fractions 10-38, Figure 32 B).

Fractions 22-38 from the S-Sepharose column were pooled, and the pool was adjusted to pH 2.2 by addition of about 11 ml 6 N HCl and applied to a Vydac C_4 column (height 8.4 cm, diameter 9 cm) equilibrated with 50% (vol/vol) ethanol, 12.5 mM HCl (solution A) and operated at 4°C. The column resin was prepared by suspending the dry resin in 80% (vol/vol) ethanol, 12.5 mM HCl (solution B) and then equilibrating it with solution A. Prior to sample application, a blank gradient from solution A to solution B (6 L total volume) was applied and the column was then re-equilibrated with solution A. After sample application, the column was washed with 2.5 L of solution A and SCF material, bound to the column, was eluted with a gradient from solution A to solution B (18 L total volume) at a flow rate of 2670 ml/h. 286 fractions of 50 ml each were collected, and aliquots were analyzed by absorbance at 280 nm (Figure 35), and by SDS-PAGE (25 µl per fraction) as described above (Figure 34 A, reducing conditions; Figure 34 B, nonreducing conditions). Fractions 62-161, containing correctly oxidized SCF in a highly purified state, were pooled [the relatively small amounts of incorrectly oxidized monomer with M_r of about 18-20,000 (unreduced) eluted later in the gradient (about fractions 166-211) and disulfide-linked dimer material also eluted later (about fractions 199-235) (Figure 35)].

To remove ethanol from the pool of fractions 62-161, and to concentrate the SCF, the following procedure utilizing Q-Sepharose Fast Flow (Pharamcia) ion exchange resin was employed. The pool (5 L) was diluted with water to a volume of 15.625 L, bringing the ethanol concentration to about 20% (vol/vol). Then 1 M Tris base (135 ml) was added to bring the pH to 8,

followed by 1 M Tris-HCl, pH 8, (23.6 ml) to bring the total Tris concentration to 10 mM. Next 10 mM Tris-HCl, pH 8 (~15.5 L) was added to bring the total volume to 31.25 L and the ethanol concentration to about 10% (vol/vol). The material was then applied at 4°C to a column of Q-Sepharose Fast Flow (height 6.5 cm, diameter 7 cm) equilibrated with 10 mM Tris-HCl, pH 8, and this was followed by washing of the column with 2.5 L of column buffer. Flow rate during sample application and wash was about 5.5 L/h. To elute the bound SCF, 200 mM NaCl, 10 mM Tris-HCl, pH 8 was pumped in reverse direction through the column at about 200 ml/h. Fractions of about 12 ml were collected and analyzed by absorbance at 280 nm, and SDS-PAGE as above. Fractions 16-28 were pooled (157 ml).

The pool containing SCF was then applied in two separate chromatographic runs (78.5 ml applied for each) to a Sephacryl S-200 HR (Pharmacia) gel filtration column (5 x 138 cm) equilibrated with phosphate-buffered saline at 4°C. Fractions of about 15 ml were collected at a flow rate of about 75 ml/h. In each case a major peak of material with absorbance at 280 nm eluted in fractions corresponding roughly to the elution volume range of 1370 to 1635 ml. The fractions representing the absorbance peaks from the two column runs were combined into a single pool of 525 ml, containing about 2.3 g of SCF. This material was sterilized by filtration using a Millipore Millipak 20 membrane cartridge.

Alternatively, material from the C_4 column can be concentrated by ultrafiltration and the buffer exchanged by diafiltration, prior to sterile filtration.

The isolated recombinant human SCF^{1-164} material is highly pure (>98% by SDS-PAGE with silver-staining) and is considered to be of pharmaceutical grade. Using the methods outlined in Example 2, it is

found that the material has amino acid composition matching that expected from analysis of the SCF gene, and has N-terminal amino acid sequence Met-Glu-Gly-Ile..., as expected, with the retention of the Met encoded by the initiation codon.

By procedures comparable to those outlined for human SCF^{1-164} expressed in E. coli, rat SCF^{1-164} (also present in insoluble form inside the cell after fermention) can be recovered in a purified state with high biological specific activity. Similarly, human SCF^{1-183} and rat SCF^{1-193} can be recovered. The rat SCF^{1-193}, during folding/oxidation, tends to form more variously oxidized species, and the unwanted species are more difficult to remove chromatographically.

The rat SCF^{1-193} and human SCF^{1-183} are prone to proteolytic degradation during the early stages of recovery, i.e., solubilization and folding/oxidation. A primary site of proteolysis is located between residues 160 and 170. The proteolysis can be minimized by appropriate manipulation of conditions (e.g., SCF concentration; varying pH; inclusion of EDTA at 2-5 mM, or other protease inhibitors), and degraded forms to the extent that they are present can be removed by appropriate fractionation steps.

While the use of urea for solubilization, and during folding/oxidation, as outlined, is a preferred embodiment, other solubilizing agents such as guanidine-HCl (e.g. 6 M during solubilization and 1.25 M during folding/oxidation) and sodium N-lauroyl sarcosine can be utilized effectively. Upon removal of the agents after folding/oxidation, purified SCFs, as determined by SDS-PAGE, can be recovered with the use of appropriate fractionation steps.

In addition, while the use of glutathione at 1 mM during folding/oxidation is a preferred embodiment, other conditions can be utilized with equal or nearly

equal effectiveness. These include, for example, the use in place of 1 mM glutathione of 2 mM glutathione plus 0.2 mM oxidized glutathione, or 4 mM glutathione plus 0.4 mM oxidized glutathione, or 1 mM 2-mercaptoethanol, or other thiol reagents also.

In addition to the chromatographic procedures described, other procedures which are useful in the recovery of SCFs in a purified active form include hydrophobic interaction chromatography [e.g., the use of phenyl-Sepharose (Pharmacia), applying the sample at neutral pH in the presence of 1.7 M ammonium sulfate and eluting with a gradient of decreasing ammonium sulfate]; immobilized metal affinity chromatography [e.g., the use of chelating-Sepharose (Pharmacia) charged with Cu^{2+} ion, applying the sample at near neutral pH in the presence of 1 mM imidazole and eluting with a gradient of increasing imidazole]; hydroxylapatite chromatography, [applying the sample at neutral pH in the presence of 1 mM phosphate and eluting with a gradient of increasing phosphate]; and other procedures apparent to those skilled in the art.

Other forms of human SCF, corresponding to all or part of the open reading frame encoding by amino acids 1-248 in Figure 42, or corresponding to the open reading frame encoded by alternatively spliced mRNAs that may exist (such as that represented by the cDNA sequence in Figure 44), can also be expressed in E. coli and recovered in purified form by procedures similar to those described in this Example, and by other procedures apparent to those skilled in the art.

The purification and formulation of forms including the so-called transmembrane region referred to in Example 16 may involve the utilization of detergents, including non-ionic detergents, and lipids, including phospholipid-containing liposome structures.

EXAMPLE 11

Recombinant SCF from Mammalian Cells

A. Fermentation of CHO Cells Producing SCF

Recombinant Chinese hamster ovary (CHO) cells (strain CHO pDSRα2 $hSCF^{1-162}$) were grown on microcarriers in a 20 liter perfusion culture system for the production of human SCF^{1-162}. The fermentor system is similar to that used for the culture of BRL 3A cells, Example 1B, except for the following: The growth medium used for the culture of CHO cells was a mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 nutrient mixture in a 1:1 proportion (GIBCO), supplemented with 2 mM glutamine, nonessential amino acids (to double the existing concentration by using 1:100 dilution of Gibco #320-1140) and 5% fetal bovine serum. The harvest medium was identical except for the omission of serum. The reactor was inoculated with 5.6×10^9 CHO cells grown in two 3-liter spinner flasks. The cells were allowed to grow to a concentration of 4×10^5 cells/ml. At this point 100 grams of presterilized cytodex-2 microcarriers (Pharmacia) were added to the reactor as a 3-liter suspension in phosphate buffered saline. The cells were allowed to attach and grow on the microcarriers for four days. Growth medium was perfused through the reactor as needed based on glucose consumption. The glucose concentration was maintained at approximately 2.0 g/L. After four days, the reactor was perfused with six volumes of serum-free medium to remove most of the serum (protein concentration <50 µg/ml). The reactor was then operated batch-wise until the glucose concentration fell below 2 g/L. From this point onward, the reactor was operated at a continuous perfusion rate of approximately 20 L/day. The pH of the culture was maintained at 6.9 ±

0.3 by adjusting the CO_2 flow rate. The dissolved oxygen was maintained higher than 20% of air saturation by supplementing with pure oxygen as necessary. The temperature was maintained at 37 ± 0.5° C.

Approximately 450 liters of serum-free conditioned medium was generated from the above system and was used as starting material for the purification of recombinant human SCF^{1-162}.

Approximately 589 liters of serum-free conditioned medium was also generated in similar fashion but using strain CHO pDSRα2 $rSCF^{1-162}$ and used as starting material for purification of rat SCF^{1-162}.

B. Purification of Recombinant Mammalian Expressed Rat SCF^{1-162}

All purification work was carried out at 4°C unless indicated otherwise.

1. Concentration and Diafiltration

Conditioned medium generated by serum-free growth of cell strain CHO pDSRα2 rat SCF^{1-162} as performed in Section A above, was clarified by filtration thru 0.45 μ Sartocapsules (Sartorius). Several different batches (36 L, 101 L, 102 L, 200 L and 150 L) were separately subjected to concentration and diafiltration/buffer exchange. To illustrate, the handling of the 36 L batch was as follows. The filtered condition medium was concentrated to ~500 ml using a Millipore Pellicon tangential flow ultrafiltration apparatus with three 10,000 molecular weight cutoff cellulose acetate membrane cassettes (15 ft^2 total membrane area; pump rate ~2,200 ml/min and filtration rate ~750 ml/min). Diafiltration/buffer exchange in preparation for anion exchange chromatography was then accomplished by adding 1000 ml of 10 mM Tris-HCl, pH

6.7-6.8 to the concentrate, reconcentrating to 500 ml using the tangential flow ultrafiltration apparatus, and repeating this 5 additional times. The concentrated/diafiltered preparation was finally recovered in a volume of 1000 ml. The behavior of all conditioned medium batches subjected to the concentration and diafiltration/buffer exchange was similar. Protein concentrations for the batches, determined by the method of Bradford [Anal. Bioch. 72, 248-254 (1976)] with bovine serum albumin as standard, were in the range 70-90 µg/ml. The total volume of conditioned medium utilized for this preparation was about 589 L.

2. Q-Sepharose Fast Flow Anion Exchange Chromatography

The concentrated/diafiltered preparations from each of the five conditioned medium batches referred to above were combined (total volume 5,000 ml). pH was adjusted to 6.75 by adding 1 M HCl. 2000 ml of 10 mM Tris-HCl, pH 6.7 was used to bring conductivity to about 0.700 mmho. The preparation was applied to a Q-Sepharose Fast Flow anion exchange column (36 x 14 cm; Pharmacia Q-Sepharose Fast Flow resin) which had been equilibrated with the 10 mM Tris-HCl, pH 6.7 buffer. After sample application, the column was washed with 28,700 ml of the Tris buffer. Following this washing the column was washed with 23,000 ml of 5 mM acetic acid/1 mM glycine/6 M urea/20 µM $CuSO_4$ at about pH 4.5. The column was then washed with 10 mM Tris-HCl, 20 µm $CuSO_4$, pH 6.7 buffer to return to neutral pH and remove urea, and a salt gradient (0-700 mM NaCl in the 10 mM Tris-HCl, 20 µM $CuSO_4$, pH 6.7 buffer; 40 L total volume) was applied. Fractions of about 490 ml were collected at a flow rate of about 3,250 ml/h. The chromatogram is shown in Figure 36. "MC/9 cpm" refers to biological activity in the MC/9 assay; 5 µl from the

indicated fractions was assayed. Eluates collected during the sample application and washes are not shown in the Figure; no biological activity was detected in these fractions.

3. Chromatography Using Silica-Bound Hydrocarbon Resin

Fractions 44-66 from the run shown in Figure 36 were combined (11,200 ml) and EDTA was added to a final concentration of 1 mM. This material was applied at a flow rate of about 2000 ml/h to a C_4 column (Vydac Proteins C_4; 7 x 8 cm) equilibrated with buffer A (10 mM Tris pH 6.7/20% ethanol). After sample application the column was washed with 1000 ml of buffer A. A linear gradient from buffer A to buffer B (10 mM Tris pH 6.7/94% ethanol) (total volume 6000 ml) was then applied, and fractions of 30-50 ml were collected. Portions of the C_4 column starting sample, runthrough pool and wash pool in addition to 0.5 ml aliquots of the gradient fractions were dialyzed against phosphate-buffered saline in preparation for biological assay. These various fractions were assayed by the MC/9 assay (5 µl aliquots of the prepared gradient fractions; cpm in Figure 37). SDS-PAGE [Laemmli, Nature 227, 680-685 (1970); stacking gels contained 4% (w/v) acrylamide and separating gels contained 12.5% (w/v) acrylamide] of aliquots of various fractions is shown in Figure 38. For the gels shown, sample aliquots (100 µl) were dried under vacuum and then redissolved using 20 µl sample treatment buffer (reducing, i.e., with 2-mercaptoethanol) and boiled for 5 min prior to loading onto the gel. The numbered marks at the left of the Figure represent migration positions of molecular weight markers (reduced) as in Figure 6. The numbered lanes represent the corresponding fractions collected during application of the last part of the gradient. The gels were silver-stained [Morrissey, Anal. Bioch. 117, 307-310 (1981)].

4. Q-Sepharose Fast Flow Anion Exchange Chromatography

Fractions 98-124 from the C_4 column shown in Figure 37 were pooled (1050 ml). The pool was diluted 1:1 with 10 mM Tris, pH 6.7 buffer to reduce ethanol concentration. The diluted pool was then applied to a Q-Sepharose Fast Flow anion exchange column (3.2 x 3 cm, Pharmacia Q-Sepharose Fast Flow resin) which had been equilibratd with the 10 mM Tris-HCl, pH 6.7 buffer. Flow rate was 463 ml/h. After sample application the column was washed with 135 ml of column buffer and elution of bound material was carried out by washing with 10 mM Tris-HCl, 350 mM NaCl, pH 6.7. The flow direction of the column was reversed in order to minimize volume of eluted material, and 7.8 ml fractions were collected during elution.

5. Sephacryl S-200 HR Gel Filtration Chromatography

Fractions containing eluted protein from the salt wash of the Q-Sepharose Fast Flow anion exchange column were pooled (31 ml). 30 ml was applied to a Sephacryl S-200 HR (Pharmacia) gel filtration column, (5 x 55.5 cm) equilibrated in phosphate-buffered saline. Fractions of 6.8 ml were collected at a flow rate of 68 ml/hr. Fractions corresponding to the peak of absorbance at 280 nm were pooled and represent the final purified material.

Table 15 shows a summary of the purification.

TABLE 15.

Summary of Purification of Mammalian Expressed Rat SCF^{1-162}

Step	Volume(ml)	Total Protein (mg)*
Conditioned medium (concentrated)	7,000	28,420
Q-Sepharose Fast Flow	11,200	974
C_4 resin	1,050	19
Q-Sepharose Fast Flow	31	20
Sephacryl S-200 HR	82	19**

*Determined by the method of Bradford (supra, 1976).

**Determined as 47.3 mg by quantitative amino acid analysis using methodology similar to that outlined in Example 2.

The N-terminal amino acid sequence of purified rat SCF^{1-162} is approximately half Gln-Glu-Ile... and half PyroGlu-Glu-Ile..., as determined by the methods outlined in Example 2. This result indicates that rat SCF^{1-162} is the product of proteolytic processing/cleavage between the residues indicated as numbers (-1) (Thr) and (+1) (Gln) in Figure 14C. Similarly, purified human SCF^{1-162} from transfected CHO cell conditioned medium (below) has N-terminal amino acid sequence Glu-Gly-Tle, indicating that it is the product of processing/cleavage between residues indicated as numbers (-1) (Thr) and (+1) (Glu) in Figure 15C.

Using the above-described protocol will yield purified human SCF protein, either recombinant forms expressed in CHO cells or naturally derived.

Additional purification methods that are of utility in the purification of mammalian cell derived recombinant SCFs include those outlined in Examples 1

and 10, and other methods apparent to those skilled in the art.

Other forms of human SCF, corresponding to all or part of the open reading frame encoded by amino acids 1-248 shown in Figure 42, or corresponding to the open reading frame encoded by alternatively spliced mRNAs that may exist (such as that represented by the cDNA sequence in Figure 44), can also be expressed in mammalian cells and recovered in purified form by procedures similar to those decribed in this Example, and by other procedures apparent to those skilled in the art.

C. SDS-PAGE and Glycosidase Treatments

SDS-PAGE of pooled fractions from the Sephacryl S-200 HR gel filtration column is shown in Figure 39; 2.5 µl of the pool was loaded (lane 1). The lane was silver-stained. Molecular weight markers (lane 6) were as described for Figure 6. The different migrating material above and slightly below the M_r 31,000 marker position represents the biologically active material; the apparent heterogeneity is largely due to the heterogeneity in glycosylation.

To characterize the glycosylation purified material was treated with a variety of glycosidases, analyzed by SDS-PAGE (reducing conditions) and visualized by silver-staining. Results are shown in Figure 39. Lane 2, neuraminidase. Lane 3, neuraminidase and O-glycanase. Lane 4, neuraminidase, O-glycanase and N-glycanase. Lane 5, neuraminidase and N-glycanase. Lane 7, N-glycanase. Lane 8, N-glycanase without substrate. Lane 9, O-glycanase without substrate. Conditions were 10 mM 3-[(3-cholamidopropyl) dimethyl ammonio]-1- propane sulfonate (CHAPS), 66.6 mM 2-mercaptoethanol, 0.04% (wt/vol) sodium azide, phosphate buffered saline, for 30 min at 37°C, followed

by incubation at half of described concentrations in presence of glycosidases for 18 h at 37°C. Neuraminidase (from Arthrobacter ureafaciens; supplied by Calbiochem) was used at 0.5 units/ml final concentration. O-Glycanase (Genzyme; endo-alpha-N-acetyl galactosaminidase) was used at 7.5 milliunits/ml. N-Glycanase (Genzyme; peptide: N-glycosidase F; peptide-N^4[N-acetyl-beta-glucosaminyl] asparagine amidase) was used at 10 units/ml.

Where appropriate, various control incubations were carried out. These included: incubation without glycosidases, to verify that results were due to the glycosidase preparations added; incubation with glycosylated proteins (e.g. glycosylated recombinant human erythropoietin) known to be substrates for the glycosidases, to verify that the glycosidase enzymes used were active; and incubation with glycosidases but no substrate, to judge where the glycosidase preparations were contributing to or obscuring the visualized gel bands (Figure 39, lanes 8 and 9).

A number of conclusions can be drawn from the experiments described above. The various treatments with N-glycanase [which removes both complex and high-mannose N-linked carbohydrate (Tarentino et al., Biochemistry 24, 4665-4671 (1988)], neuraminidase (which removes sialic acid residues), and O-glycanase [which removes certain O-linked carbohydrates (Lambin et al., Biochem. Soc. Trans. 12, 599-600 (1984)], suggest that: both N-linked and O-linked carbohydrates are present; and sialic acid is present, with at least some of it being part of the O-linked moieties. The fact that treatment with N-glycanase can convert the heterogeneous material apparent by SDS-PAGE to a faster-migrating form which is much more homogeneous indicates that all of the material represents the same polypeptide, with the heterogeneity being caused mainly by heterogeneity in glycosylation.

EXAMPLE 12

Preparation of Recombinant SCF^{1-164}PEG

Rat SCF^{1-164}, purified from a recombinant *E. coli* expression system according to Examples 6A and 10, was used as starting material for polyethylene glycol modification described below.

Methoxypolyethylene glycol-succinimidyl succinate (18.1 mg = 3.63 umol; SS-MPEG = Sigma Chemical Co. no. M3152, approximate molecular weight = 5,000) in 0.327 mL deionized water was added to 13.3 mg (0.727 umol) recombinant rat SCF^{1-164} in 1.0 mL 138 mM sodium phosphate, 62 mM NaCl, 0.62 mM sodium acetate, pH 8.0. The resulting solution was shaken gently (100 rpm) at room temperature for 30 minutes. A 1.0 mL aliquot of the final reaction mixture (10 mg protein) was then applied to a Pharmacia Superdex 75 gel filtration column (1.6 x 50 cm) and eluted with 100 mM sodium phosphate, pH 6.9, at a rate of 0.25 mL/min at room temperature. The first 10 mL of column effluent were discarded, and 1.0 mL fractions were collected thereafter. The UV absorbance (280 nm) of the column effluent was monitored continuously and is shown in Figure 40A. Fractions number 25 through 27 were combined and sterilized by ultrafiltration through a 0.2 μ polysulfone membrane (Gelman Sciences no. 4454), and the resulting pool was designated PEG-25. Likewise, fractions number 28 through 32 were combined, sterilized by ultrafiltration, and designated PEG-32. Pooled fraction PEG-25 contained 3.06 mg protein and pooled fraction PEG-32 contained 3.55 mg protein, as calculated from A280 measurements using for calibration an absorbance of 0.66 for a 1.0 mg/mL solution of unmodified rat SCF^{1-164}. Unreacted rat SCF^{1-164}, representing 11.8% of the total protein in the reaction mixture, was eluted in fractions number 34 to 37. Under similar chromatographic conditions,

unmodified rat SCF^{1-164} was eluted as a major peak with a retention volume of 45.6 mL, Figure 40B. Fractions number 77 to 80 in Figure 40A contained N-hydroxysuccinimide, a by-product of the reaction of rat SCF^{1-164} with SS-MPEG.

Potentially reactive amino groups in rat SCF^{1-164} include 12 lysine residues and the alpha amino group of the N-terminal glutamine residue. Pooled fraction PEG-25 contained 9.3 mol of reactive amino groups per mol of protein, as determined by spectroscopic titration with trinitrobenzene sulfonic acid (TNBS) using the method described by Habeeb, Anal. Biochem. 14:328-336 (1966). Likewise, pooled fraction PEG-32 contained 10.4 mol and unmodified rat SCF^{1-164} contained 13.7 mol of reactive amino groups per mol of protein, respectively. Thus, an average of 3.3 (13.7 minus 10.4) amino groups of rat SCF^{1-164} in pooled fraction PEG-32 were modified by reaction with SS-MPEG. Similarly, an average of 4.4 amino groups of rat SCF^{1-164} in pooled fraction PEG-25 were modified. Human SCF ($hSCF^{1-164}$) produced as in Example 10 was also modified using the procedures noted above. Specifically, 714 mg (38.5 umol) $hSCF^{1-164}$ were reacted with 962.5 mg (192.5 umol) SS-MPEG in 75 mL of 0.1 M sodium phosphate buffer, pH 8.0 for 30 minutes at room temperature. The reaction mixture was applied to a Sephacryl S-200HR column (5 x 134 cm) and eluted with PBS (Gibco Dulbecco's phosphate-buffered saline without $CaCl_2$ and $MgCl_2$) at a rate of 102 mL/hr, and 14.3-mL fractions were collected. Fractions no. 39-53, analogous to the PEG-25 pool described above and in Figure 40A, were pooled and found to contain a total of 354 mg of protein. In vivo activity of this modified SCF in primates is presented in Example 8C.

EXAMPLE 13

SCF Receptor Expression on Leukemic Blasts

Leukemic blasts were harvested from the peripheral blood of a patient with a mixed lineage leukemia. The cells were purified by density gradient centrifugation and adherence depletion. Human SCF^{1-164} was iodinated according to the protocol in Example 7. The cells were incubated with different concentrations of iodinated SCF as described [Broudy, Blood, 75 1622-1626 (1990)]. The results of the receptor binding experiment are shown in Figure 41. The receptor density estimated is approximately 70,000 receptors/cell.

EXAMPLE 14

Rat SCF Activity on Early Lymphoid Precursors

The ability of recombinant rat SCF^{1-164} ($rrSCF^{1-164}$), to act synergistically with IL-7 to enhance lymphoid cell proliferation was studied in agar cultures of mouse bone marrow. In this assay, the colonies formed with $rrSCF^{1-164}$ alone contained monocytes, neutrophils, and blast cells, while the colonies stimulated by IL-7 alone or in combination with $rrSCF^{1-164}$ contained primarily pre-B cells. Pre-B cells, characterized as $B220^+$, sIg^-, $c\mu^+$, were identified by FACS analysis of pooled cells using fluorescence-labeled antibodies to the B220 antigen [Coffman, Immunol. Rev., 69, 5 (1982)] and to surface Ig (FITC-goat anti-K, Southern Biotechnology Assoc., Birmingham, AL); and by analysis of cytospin slides for cytoplasmic μ expression using fluorescence-labeled antibodies (TRITC-goat anti-μ, Southern Biotechnology Assoc.,). Recombinant human IL-7 (rhIL-7) was obtained from Biosource International (Westlake Village, CA). When $rrSCF^{1-164}$ was added in combination with the pre-B

cell growth factor IL-7, a synergistic increase in colony formation was observed (Table 16), indicating a stimulatory role of $rrSCF^{1-164}$ on early B cell progenitors.

Table 16. Stimulation of Pre-B Cell Colony Formation by $rrSCF^{1-164}$ in Combination with hIL-7

Growth Factors				Colony Number[1]
Saline				0
$rrSCF^{1-164}$	200 ng			13 ± 2
	100 ng			7 ± 4
	50 ng			4 ± 2
rhIL-7	200 ng			21 ± 6
	100 ng			18 ± 6
	50 ng			13 ± 6
	25 ng			4 ± 2
rhIL-7	200 ng +	$rrSCF^{1-164}$	200 ng	60 ± 0
	100 ng +		200 ng	48 ± 8
	50 ng +		200 ng	24 ± 10
	25 ng +		200 ng	21 ± 2

[1] Number of colonies per 5×10^4 mouse bone marrow cells plated.

Each value is the mean of triplicate dishes ± SD.

EXAMPLE 15

Identification of the Receptor for SCF

A. c-kit is the Receptor for SCF^{1-164}

To test whether SCF^{1-164} is the ligand for c-kit, the cDNA for the entire murine c-kit [Qiu et al., EMBO J., 7, 1003-1011 (1988)] was amplified using PCR from the SCF^{1-164} responsive mast cell line MC/9 [Nabel et al., Nature, 291, 332-334 (1981)] with primers designed from the published sequence. The ligand binding and transmembrane domains of human c-kit, encoded by amino acids 1-549 [Yarden et al., EMBO J., 6, 3341-3351 (1987)], were cloned using similar techniques from the human erythroleukemia cell line, HEL [Martin and Papayannopoulou, Science, 216, 1233-1235 (1982)]. The c-kit cDNAs were inserted into the mammalian expression vector V19.8 transfected into COS-1 cells, and membrane fractions prepared for binding assays using either rat or human ^{125}I-SCF^{1-164} according to the methods described in Sections B and C below. Table 17 shows the data from a typical binding assay. There was no detectable specific binding of ^{125}I human SCF^{1-164} to COS-1 cells transfected with V19.8 alone. However, COS-1 cells expressing human recombinant c-kit ligand binding plus transmembrane domains (hckit-LT1) did bind ^{125}I-$hSCF^{1-164}$ (Table 17). The addition of a 200 fold molar excess of unlabelled human SCF^{1-164} reduced binding to background levels. Similarly, COS-1 cells transfected with the full length murine c-kit (mckit-L1) bound rat ^{125}I-SCF^{1-164}. A small amount of rat ^{125}I-SCF^{1-164} binding was detected in COS-1 cells transfectants with V19.8 alone, and has also been observed in untransfected cells (not shown), indicating that COS-1 cells express endogenous c-kit. This finding is in accord with the broad cellular distribution of

c-kit expression. Rat ^{125}I-SCF$^{1-164}$ binds similarly to both human and murine c-kit, while human ^{125}I-SCF$^{1-164}$ bind with lower activity to murine c-kit (Table 17). This data is consistent with the pattern of SCF$^{1-164}$ cross-reactivity between species. Rat SCF$^{1-164}$ induces proliferation of human bone marrow with a specific activity similar to that of human SCF$^{1-164}$, while human SCF$^{1-164}$ induced proliferation of murine mast cells occurs with a specific activity 800 fold less than the rat protein.

In summary, these findings confirm that the phenotypic abnormalities expressed by W or Sl mutant mice are the consequences of primary defects in c-kit receptor/ligand interactions which are critical for the development of diverse cell types.

Table 17. SCF$^{1-164}$ Binding to Recombinant c-kit Expressed in COS-1 Cells.

Plasmid Transfected	CPM Bound[a] Human SCF$^{1-164}$ ^{125}I-SCF[b]	^{125}I-SCF+cold[c]	Rat SCF$^{1-164}$ ^{125}I-SCF[d]	^{125}I-SCF+cold[e]
V19.8	2,160	2,150	1,100	550
V19.8:hckit-LT1	59,350	2,380	70,000	1,100
V19.8:mckit-L1	9,500	1,100	52,700	600

[a] The average of duplicate measurements is shown; the experiment has been independently performed with similar results three times.
[b] 1.6 nM human ^{125}I-SCF$^{1-164}$
[c] 1.6 nM human ^{125}I-SCF$^{1-164}$ + 320 nM unlabelled human SCF$^{1-164}$
[d] 1.6 nM rat ^{125}I-SCF$^{1-164}$
[e] 1.6 nM rat ^{125}I-SCF$^{1-164}$ + 320 nM unlabelled rat SCF$^{1-164}$

B. Recombinant c-kit Expression in COS-1 Cells

Human and murine c-kit cDNA clones were derived using PCR techniques [Saiki et al., Science, 239, 487-491 (1988)] from total RNA isolated by an acid phenol/chloroform extraction procedure [Chomczynsky and Sacchi, Anal. Biochem., 162, 156-159, (1987)] from the human erythroleukemia cell line HEL and MC/9 cells, respectively. Unique sequence oligonucleotides were designed from the published human and murine c-kit sequences. First strand cDNA was synthesized from the total RNA according to the protocol provided with the enzyme, Mo-MLV reverse transcription (Bethesda Research Laboratories, Bethesda, MD), using c-kit antisense oligonucleotides as primers. Amplification of overlapping regions of the c-kit ligand binding and tyrosine kinase domains was accomplished using appropriate pairs of c-kit primers. These regions were cloned into the mammalian expression vector V19.8 (Figure 17) for expression in COS-1 cells. DNA sequencing of several clones revealed independent mutations, presumably arising during PCR amplification, in every clone. A clone free of these mutations was constructed by reassembly of mutation-free restriction fragments from separate clones. Some differences from the published sequence appeared in all or in about half of the clones; these were concluded to be the actual sequences present in the cell lines used, and may represent allelic differences from the published sequences. The following plasmids were constructed in V19.8: V19.8:mckit-LT1, the entire murine c-kit; and V19.8:hckit-L1, containing the ligand binding plus transmembrane region (amino acids 1-549) of human c-kit.

The plasmids were transfected into COS-1 cells essentially as described in Example 4.

C. ^{125}I-SCF^{1-164} Binding to COS-1 Cells Expressing Recombinant c-kit

Two days after transfection, the COS-1 cells were scraped from the dish, washed in PBS, and frozen until use. After thawing, the cells were resuspended in 10 mM Tris-HCl, 1 mM $MgCl_2$ containing 1 mM PMSF, 100 µg/ml aprotinin, 25 µg/ml leupeptin, 2 µg/ml pepstatin, and 200 µg/ml TLCK-HCl. The suspension was dispersed by pipetting up and down 5 times, incubated on ice for 15 minutes, and the cells were homogenized with 15-20 strokes of a Dounce homogenizer. Sucrose (250mM) was added to the homogenate, and the nuclear fraction and residual undisrupted cells were pelleted by centrifugation at 500 x g for 5 min. The supernatant was centrifuged at 25,000 g for 30 min. at 4°C to pellet the remaining cellular debris. Human and rat SCF^{1-164} were radioiodinated using chloramine-T [Hunter and Greenwood, Nature, 194, 495-496 (1962)]. COS-1 membrane fractions were incubated with either human or rat ^{125}I-SCF^{1-164} (1.6nM) with or without a 200 fold molar excess of unlabelled SCF^{1-164} in binding buffer consisting of RPMI supplemented with 1% bovine serum albumin and 50 mM HEPES (pH 7.4) for 1 h at 22°C. At the conclusion of the binding incubation, the membrane preparations were gently layered onto 150 µl of phthalate oil and centrifuged for 20 minutes in a Beckman Microfuge 11 to separate membrane bound ^{125}I-SCF^{1-164} from free ^{125}I-SCF^{1-164}. The pellets were clipped off and membrane associated ^{125}I-SCF^{1-164} was quantitated.

EXAMPLE 16

Isolation of a Human SCF cDNA

A. Construction of the HT-1080 cDNA Library

Total RNA was isolated from human fibrosarcoma cell line HT-1080 (ATCC CCL 121) by the acid guanidinium thiocyanate-phenol-chloroform extraction method [Chomczynski et al., Anal. Biochem. 162, 156 (1987)], and poly(A) RNA was recovered by using oligo(dT) spin column purchased from Clontech. Double-stranded cDNA was prepared from 2 µg poly(A) RNA with a BRL (Bethesda Research Laboratory) cDNA synthesis kit under the conditions recommended by the supplier. Approximately 100ng of column fractionated double-stranded cDNA with an average size of 2kb was ligated to 300ng SalI/NotI digested vector pSPORT 1 [D'Alessio et al., Focus, 12, 47-50 (1990)] and transformed into DH5α (BRL, Bethesda, MD) cells by electroporation [Dower et al., Nucl. Acids Res., 16, 6127-6145 (1988)].

B. Screening of the cDNA Library

Approximately 2.2 x 10^5 primary transformants were divided into 44 pools with each containing ~5000 individual clones. Plasmid DNA was prepared from each pool by the CTAB-DNA precipitation method as described [Del Sal et al., Biotechniques, 7, 514-519 (1989)]. Two micrograms of each plasmid DNA pool was digested with restriction enzyme NotI and separated by gel electrophoresis. Linearized DNA was transferred onto GeneScreen Plus membrane (DuPont) and hybridized with ^{32}P-labeled PCR generated human SCF cDNA (Example 3) under conditions previously described [Lin et al., Proc. Natl. Acad. Sci. USA, 82, 7580-7584 (1985)]. Three pools containing positive signal were identified from

the hybridization. These pools of colonies were rescreened by the colony-hybridization procedure [Lin et al., Gene 44, 201-209 (1986)] until a single colony was obtained from each pool. The cDNA sizes of these three isolated clones are between 5.0 to 5.4 kb. Restriction enzyme digestions and nucleotide sequence determination at the 5' end indicate that two out of the three clones are identical (10-1a and 21-7a). They both contain the coding region and approximately 200bp of 5' untranslated region (5'UTR). The third clone (26-1a) is roughly 400bp shorter at the 5' end than the other two clones. The sequence of this human SCF cDNA is shown in Figure 42. Of particular note is the hydrophobic transmembrane domain sequence starting in the region of amino acids 186-190 and ending at amino acid 212.

C. Construction of pDSRα2 $hSCF^{1-248}$

pDSRα2 $hSCF^{1-248}$ was generated using plasmids 10-1a (as described in Example 16B) and pGEM3 $hSCF^{1-164}$ as follows: The HindIII insert from pGEM3 $hSCF^{1-164}$ was transferred to M13mp18. The nucleotides immediately upstream of the ATG initiation codon were changed by site directed mutagenesis from tttccttATG to gccgccgccATG using the antisense oligonucleotide

5'-TCT TCT TCA TGG CGG CGG CAA GCT T 3'

and the oligonucleotide-directed in vitro mutagenesis system kit and protocols from Amersham Corp. to generate M13mp18 $hSCF^{K1-164}$. This DNA was digested with HindIII and inserted into pDSRα2 which had been digested with HindIII. This clone is designated pDSRα2 $hSCF^{K1-164}$. DNA from pDSRα2 $hSCF^{K1-164}$ was digested with XbaI and the DNA made blunt ended by the addition of Klenow enzyme and four dNTPs. Following termination of this reaction the DNA was further digested with the enzyme SpeI. Clone 10-1a was digested with DraI to

generate a blunt end 3' to the open reading frame in the insert and with SpeI which cuts at the same site within the gene in both pDSRα2 $hSCF^{K1-164}$ and 10-1a. These DNAs were ligated together to generate pDSRα2 $hSCF^{K1-248}$.

D. Transfection and immunoprecipitation of COS cells with pDSRα2 $hSCF^{K1-248}$ DNA.

COS-7 (ATCC CRL 1651) cells were transfected with DNA constructed as described above. $4x10^6$ cells in 0.8 ml DMEM + 5% FBS were electroporated at 1600 V with either 10 µg pDSRα2 $hSCF^{K1-248}$ DNA or 10 µg pDSRα2 vector DNA (vector control). Following electroporation, cells were replated into two 60-mm dishes. After 24 hrs, the medium was replaced with fresh complete medium.

72 hrs after transfection, each dish was labelled with ^{35}S-medium according to a modification of the protocol of Yarden et al. (PNAS 87, 2569-2573, 1990). Cells were washed once with PBS and then incubated with methionine-free, cysteine-free DMEM (met^-cys^-DMEM) for 30 min. The medium was removed and 1 ml met^-cys^- DMEM containing 100 µCi/ml Tran^{35}S-Label (ICN) was added to each dish. Cells were incubated at 37°C for 8 hr. The medium was harvested, clarified by centrifugation to remove cell debris and frozen at -20°C.

Aliquots of labelled conditioned medium of COS/pDSRα2 $hSCF^{K1-248}$ and COS/pDSRα2 vector control were immunoprecipitated along with medium samples of ^{35}S-labelled CHO/pDSRα2 $hSCF^{1-164}$ clone 17 cells (see Example 5) according to a modification of the protocol of Yarden et al. (EMBO, J., 6, 3341-3351, 1987). One ml of each sample of conditioned medium was treated with 10 µl of pre-immune rabbit serum (#1379 P.I.). Samples

were incubated for 5 h. at 4°C. One hundred microliters of a 10% suspension of Staphylococcus aureus (Pansorbin, Calbiochem.) in 0.15 M NaCl, 20 mM Tris pH 7.5, 0.2% Triton X-100 was added to each tube. Samples were incubated for an additional one hour at 4°C. Immune complexes were pelleted by centrifugation at 13,000 x g for 5 min. Supernatants were transferred to new tubes and incubated with 5 µl rabbit polyclonal antiserum (#1381 TB4), purified as in Example 11, against CHO derived $hSCF^{1-162}$ overnight at 4°C. 100 µl Pansorbin was added for 1 h. and immune complexes were pelleted as before. Pellets were washed 1x with lysis buffer (0.5% Na-deoxycholate, 0.5% NP-40, 50mM NaCl, 25 mM Tris pH 8), 3x with wash buffer (0.5 M NaCl, 20 mM Tris pH 7.5, 0.2% Triton X-100), and 1x with 20 mM Tris pH 7.5. Pellets were resuspended in 50 µl 10 mM Tris pH 7.5, 0.1% SDS, 0.1 M ß-mercaptoethanol. SCF protein was eluted by boiling for 5 min. Samples were centrifuged at 13,000 x g for 5 min. and supernatants were recovered.

Treatment with glycosidases was accomplished as follows: three microliters of 75 mM CHAPS containing 1.6 mU O-glycanase, 0.5 U N-glycanase, and 0.02 U neuraminidase was added to 25 µl of immune complex samples and incubated for 3 hr. at 37°C. An equal volume of 2xPAGE sample buffer was added and samples were boiled for 3 min. Digested and undigested samples were electrophoresed on a 15% SDS-polyacrylamide reducing gel overnight at 8 mA. The gel was fixed in methanol-acetic acid, treated with Enlightening enhancer (NEN) for 30 min., dried, and exposed to Kodak XAR-5 film at -70°.

Figure 43 shows the autoradiograph of the results. Lanes 1 and 2 are samples from control COS/pDSRα2 cultures, lanes 3 and 4 from $COS/pSR\alpha 2hSCF^{K1-248}$, lanes 5 and 6 from CHO/pDSRα2

$hSCF^{1-164}$. Lanes 1, 3, and 5 are undigested immune precipitates; lanes 2, 4, and 6 have been digested with glycanases as described above. The positions of the molecular weight markers are shown on the left. Processing of the SCF in COS transfected with pDSRα2 $hSCF^{K1-248}$ closely resembles that of $hSCF^{1-164}$ secreted from CHO transfected with pDSRα2 $hSCF^{1-164}$, (Example 11). This strongly suggests that the natural proteolytic processing site releasing SCF from the cell is in the vicinity of amino acid 164.

EXAMPLE 17

Quaternary Structure Analysis of Human SCF.

Upon calibration of the gel filtration column (ACA 54) described in Example 1 for purification of SCF from BRL cell medium with molecular weight standards, and upon elution of purified SCF from other calibrated gel filtration columns, it is evident that SCF purified from BRL cell medium behaves with an apparent molecular weight of approximately 70,000-90,000 relative to the molecular weight standards. In contrast, the apparent molecular weight by SDS-PAGE is approximately 28,000-35,000. While it is recognized that glycosylated proteins may behave anomalously in such analyses, the results suggest that the BRL-derived rat SCF may exist as non-covalently associated dimer under non-denaturing conditions. Similar results apply for recombinant SCF forms (e.g. rat and human SCF^{1-164} derived from *E. coli*, rat and human SCF^{1-162} derived from CHO cells) in that the molecular size estimated by gel filtration under non-denaturing conditions is roughly twice that estimated by gel filtration under denaturing conditions (i.e., presence of SDS), or by SDS-PAGE, in each particular case. Furthermore sedimentation velocity analysis, which provides an accurate determination of

molecular weight in solution, gives a value of about 36,000 for molecular weight of E. coli-derived recombinant human SCF^{1-164}. This value is again approximately twice that seen by SDS-PAGE (~18,000-19,000). Therefore, while it is recognized that there may be multiple oligomeric states (including the monomeric state), it appears that the dimeric state predominates under some circumstances in solution.

EXAMPLE 18
Isolation of Human SCF cDNA Clones from the 5637 Cell Line

A. Construction of the 5637 cDNA Library

Total RNA was isolated from human bladder carcinoma cell line 5637 (ATCC HTB-9) by the acid guanidinium thiocyanate-phenol-chloroform extraction method [Chomczynski et al., Anal. Biochem, 162, 156 (1987)], and poly(A) RNA was recovered by using an oligo(dT) spin column purchased from Clontech. Double-stranded cDNA was prepared from 2 µg poly(A) RNA with a BRL cDNA synthesis kit under the conditions recommended by the supplier. Approximately 80 ng of column fractionated double-stranded cDNA with an average size of 2 kb was ligated to 300 ng SalI/NotI digested vector pSPORT 1 [D'Alessio et al., Focus, 12, 47-50 (1990)] and transformed into DH5α cells by electroporation [Dower et al., Nucl. Acids Res., 16, 6127-6145 (1988)].

B. Screening of the cDNA Library

Approximately 1.5×10^5 primary tranformants were divided into 30 pools with each containing approximately 5000 individual clones. Plasmid DNA was prepared from each pool by the CTAB-DNA precipitation

method as described [Del Sal et al., Biotechniques, 7, 514-519 (1989)]. Two micrograms of each plasmid DNA pool was digested with restriction enzyme NotI and separated by gel electrophoresis. Linearized DNA was transferred to GeneScreen Plus membrane (DuPont) and hybridized with ^{32}P-labeled full length human SCF cDNA isolated from HT1080 cell line (Example 16) under the conditions previously described [Lin et al., Proc. Natl. Acad. Sci. USA, 82, 7580-7584 (1985)]. Seven pools containing positive signal were identified from the hybridization. The pools of colonies were rescreened with ^{32}P-labeled PCR generated human SCF cDNA (Example 3) by the colony hybridization procedure [Lin et al., Gene, 44, 201-209 (1986)] until a single colony was obtained from four of the pools. The insert sizes of four isolated clones are approximately 5.3 kb. Restriction enzyme digestions and nucleotide sequence analysis of the 5'-ends of the clones indicate that the four clones are identical. The sequence of this human cDNA is shown in Figure 44. The cDNA of Figure 44 codes for a polypeptide in which amino acids 149-177 of the sequences in Figure 42 are replaced by a single Gly residue.

EXAMPLE 19

SCF Enhancement of Survival After Lethal Irradiation.

A. SCF in vivo activity on Survival After Lethal Irradiation.

The effect of SCF on survival of mice after lethal irradiation was tested. Mice used were 10 to 12 week-old female Balb/c. Groups of 5 mice were used in all experiments and the mice were matched for body weight within each experiment. Mice were irradiated at

850 rad or 950 rad in a single dose. Mice were injected with factors alone or factors plus normal Balb/c bone marrow cells. In the first case, mice were injected intravenously 24 hrs. after irradiation with rat PEG-SCF$^{1-164}$ (20 µg/kg), purified from E. coli and modified by the addition of polyethylene glycol as in Example 12, or with saline for control animals. For the transplant model, mice were injected i.v. with various cell doses of normal Balb/c bone marrow 4 hours after irradiation. Treatment with rat PEG-SCF$^{1-164}$ was performed by adding 200 µg/kg of rat PEG-SCF$^{1-164}$ to the cell suspension 1 hour prior to injection and given as a single i.v. injection of factor plus cells.

After irradiation at 850 rads, mice were injected with rat PEG-SCF$^{1-164}$ or saline. The results are shown in Figure 45. Injection of rat PEG-SCF$^{1-164}$ significantly enhanced the survival time of mice compared to control animals ($P<0.0001$). Mice injected with saline survived an average of 7.7 days, while rat PEG-SCF$^{1-164}$ treated mice survived an average of 9.4 days (Figure 45). The results presented in Figure 45 represent the compilation of 4 separate experiments with 30 mice in each treatment group.

The increased survival of mice treated with rat PEG-SCF$^{1-164}$ suggests an effect of SCF on the bone marrow cells of the irradiated animals. Preliminary studies of the hematological parameters of these animals show slight increases in platelet levels compared to control animals at 5 days post irradiation, however at 7 days post irradiation the platelet levels are not significantly different to control animals. No differences in RBC or WBC levels or bone marrow cellularity have been detected.

B. Survival of transplanted mice treated with SCF

Doses of 10% femur of normal Balb/c bone marrow cells transplanted into mice irradiated at 850 rad can rescue 90% or greater of animals (data not presented). Therefore a dose of irradiation of 850 rad was used with a transplant dose of 5% femur to study the effects of rat PEG-SCF$^{1-164}$ on survival. At this cell dose it was expected that a large percentage of mice not receiving SCF would not survive; if rat PEG-SCF$^{1-164}$ could stimulate the transplanted cells there might be an increase in survival. As shown in Figure 46, approximately 30% of control mice survived past 8 days post irradiation. Treatment with rat PEG-SCF$^{1-164}$ resulted in a dramatic increase of survival with greater than 95% of these mice surviving out to at least 30 days (Figure 46). The results presented in Figure 46 represent the compilation of results from 4 separate experiments representing 20 mice in both the control and rat PEG-SCF$^{1-164}$ treated mice. At higher doses of irradiation, treatment of mice with rat PEG-SCF$^{1-164}$ in conjunction with marrow transplant also resulted in increased survival (Figure 47). Control mice irradiated at 950 rads and transplanted with 10% of a femur were dead by day 8, while approximately 40% of mice treated with rat PEG-SCF$^{1-164}$ survived 20 days or longer. 20% of control mice transplanted with 20% of a femur survived past 20 days while 80% of rSCF treated animals survived (Figure 47).

EXAMPLE 20

Production of Monoclonal Antibodies Against SCF

8-week old female BALB/c mice (Charles River, Wilmington, MA) were injected subcutaneously with 20 µg of human SCF$^{1-164}$ expressed from E. coli in complete

Freund's adjuvant (H37-Ra; Difco Laboratories, Detroit, MI). Booster immunizations of 50 µg of the same antigen in Incomplete Freund's adjuvant were subsequently administered on days 14,38 and 57. Three days after the last injection, 2 mice were sacrificed and their spleen cells fused with the sp 2/0 myeloma line according to the procedures described by Nowinski et al., [Virology 93, 111-116 (1979)].

The media used for cell culture of sp 2/0 and hybridoma was Dulbecco's Modified Eagle's Medium (DMEM), (Gibco, Chagrin Falls, Ohio) supplemented with 20% heat inactivated fetal bovine serum (Phibro Chem., Fort Lee, NJ), 110 mg/ml sodium pyruvate, 100 U/ml penicillin and 100 mcg/ml streptomycin (Gibco). After cell fusion hybrids were selected in HAT medium, the above medium containing 10^{-4}M hypoxanthine, $4x10^{-7}$M aminopterin and $1.6x10^{-5}$M thymidine, for two weeks, then cultured in media containing hypoxanthine and thymidine for two weeks.

Hybridomas were screened as follows: Polystyrene wells (Costar, Cambridge, MA) were sensitized with 0.25 µg of human SCF^{1-164} (E. coli) in 50 µl of 50 mM bicarbonate buffer pH 9.2 for two hours at room temperature, then overnight at 4°C. Plates were then blocked with 5% BSA in PBS for 30 minutes at room temperature, then incubated with hybridoma culture supernatant for one hour at 37°C. The solution was decanted and the bound antibodies incubated with a 1:500 dilution of Goat-anti-mouse IgG conjugated with Horse Radish Peroxidase (Boehringer Mannheim Biochemicals, Indianapolis, IN) for one hour at 37°C. The plates were washed with wash solution (KPL, Gaithersburg, MD) then developed with mixture of H_2O_2 and ABTS (KPL). Colorimetry was conducted at 405 nm.

Hybridoma cell cultures secreting antibody specific for human SCF^{1-164} (E coli) were tested by

ELISA, same as hybridoma screening procedures, for crossreactivities to human SCF^{1-162} (CHO). Hybridomas were subcloned by limiting dilution method. 55 wells of hybridoma supernatant tested strongly positive to human SCF^{1-164} (*E. coli*); 9 of them crossreacted to human SCF^{1-162} (CHO).

Several hybridoma cells have been cloned as follows:

Monoclone	IgG Isotype	Reactivity to human SCF^{1-162} (CHO)
4G12-13	IgG1	No
6C9A	IgG1	No
8H7A	IgG1	Yes

Hybridomas 4G12-13 and 8H7A were deposited with the ATCC on September 26, 1990.

* * *

While the present invention has been described in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

The features disclosed in the foregoing description, in the following claims and/or in the accompanying drawings may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.

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Hu164SCF17 was deposited with ATCC on 25 September 1990 under number CLR10557, 4G-12-13 was deposited with ATCC on 26 September 1990 under number HB10561, 8H7A was deposited with ATCC on 26 September 1990 under number HB 10560, V19.8 was deposited with ATCC on 13 October 1989 under number 68124 and HuGenl-Human SCF was deposited on 13 October 1989 under number 40681.



CLAIMS

1. A non-naturally-occurring polypeptide having an amino acid sequence sufficiently duplicative of that of naturally-occurring stem cell factor to allow possession of a hematopoietic biological activity of naturally occurring stem cell factor.

2. A purified polypeptide comprising naturally-occurring stem cell factor.

3. A polypeptide according to Claim 1 or 2 wherein said polypeptide is the product of procaryotic or eukaryotic expression of an exogenous DNA sequence.

4. A polypeptide according to Claim 3 wherein said polypeptide is the product of CHO cell expression.

5. A polypeptide according to Claim 3 wherein the exogenous DNA sequence is a cDNA sequence.

6. A polypeptide according to Claim 1 or 2 wherein said stem cell factor is human stem cell factor.

7. A polypeptide according to Claim 3 wherein the exogenous DNA sequence is a genomic DNA sequence.

8. A polypeptide according to Claim 3 wherein the exogenous DNA sequence is carried on an autonomously replicating DNA plasmid or viral vector.

9. A polypeptide according to Claim 1 possessing part or all of the amino acid sequence of human stem cell factor as set forth in Figure 15B, Figure 15C, Figure 42 or Figure 44 or any naturally-occurring allelic variant thereof.

10. A polypeptide according to Claim 1 which has an in vivo biological activity of naturally-occurring stem cell factor.

11. A polypeptide according to Claim 1 which has an in vitro biological activity of naturally-occurring stem cell factor.

12. A polypeptide according to Claim 1 or 2 further characterized by being covalently associated with a detectable label substance.

13. An isolated DNA sequence for use in securing expression in a procaryotic or eukaryotic host cell of a polypeptide product having an amino acid sequence sufficiently duplicative of that of naturally-occurring stem cell factor to allow possession of a hematopoietic biological activity of naturally occurring stem cell factor, said DNA sequence selected from among:

(a) DNA sequences set out in Figure 14B, Figure 14C, Figure 15B, Figure 15C, Figure 42, Figure 44, or their complementary strands;

(b) DNA sequences which hybridize to the DNA sequences defined in (a) or fragments thereof; and

(c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) and (b).

14. A procaryotic or eukaryotic host cell transformed or transfected with a DNA sequence according to Claim 13 in a manner allowing the host cell to express said polypeptide product.

15. A polypeptide product of the expression of a DNA sequence of Claim 13 in a procaryotic or eukaryotic host cell.

16. An isolated DNA sequence coding for procaryotic or eukaryotic host expression of a polypeptide having an amino acid sequence sufficiently duplicative of that of naturally occurring stem cell factor to allow possession of a hematopoietic biological activity of naturally-occurring stem cell factor.

17. A cDNA sequence according to Claim 16.

18. A genomic DNA sequence according to Claim 16.

19. A DNA sequence according to Claim 16 wherein said DNA sequence codes for human stem cell factor.

20. A DNA sequence according to Claim 19 and including one or more codons preferred for expression in E. coli cells.

21. A DNA sequence according to Claim 16 having the sequence set out in Figure 15B, Figure 15C, Figure 42 or Figure 44.

22. A DNA sequence according to Claim 16 and including one or more codons preferred for expression in yeast cells.

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23. A DNA sequence according to Claim 16 covalently associated with a detectable label substance.

24. A DNA sequence coding for a polypeptide fragment or polypeptide analog of naturally-occurring stem cell factor.

25. A DNA sequence as in Claim 24 coding for methionyl stem cell factor.

26. A biologically functional plasmid or viral DNA vector including a DNA sequence according to Claim 16.

27. A procaryotic or eukaryotic host cell stably transformed or transfected with a DNA vector according to Claim 26.

28. A polypeptide product of the expression in a procaryotic or eukaryotic host cell of a DNA sequence according to Claim 16.

29. A polypeptide having part or all of the amino acid sequence as set forth in Figure 15C, Figure 42 or Figure 44 and having one or more of the in vitro biological activities of naturally-occurring stem cell factor.

30. A polypeptide having part or all of the secondary conformation of naturally-occurring stem cell factor and having part or all of the amino acid sequence set forth in Figure 15C, Figure 42, or Figure 44 and having a biological property of naturally-occurring human stem cell factor.

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31. A process for the production of stem cell factor comprising:

growing, under suitable nutrient conditions, procaryotic or eukaryotic host cells transformed or transfected with a DNA according to Claim 13, and

isolating desired polypeptide products of the expression of DNA sequences in said vector.

32. A composition comprising a purified and isolated human stem cell factor free of association with any human protein in glycosylated or nonglycosylated form.

33. A pharmaceutical composition comprising an effective amount of a polypeptide according to Claim 1 and a pharmaceutically-acceptable diluent, adjuvant or carrier.

34. A method for treatment of leucopenia in a mammal comprising administering a therapeutically effective amount of the polypeptide according to Claim 1.

35. A method for treatment of thrombocytopenia in a mammal comprising administering a therapeutically effective amount of the polypeptide according to Claim 1.

36. A method for treating anemia in a mammal comprising administering a therapeutically effective amount of the polypeptide according to Claim 1.

37. A method for enhancing engraftment of bone marrow during transplantation in a mammal comprising administering a therapeutically effective amount of the polypeptide according to Claim 1.

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38. A method of enhancing bone marrow recovery in treatment of radiation, chemical, or chemotherapeutic induced bone marrow aplasia or myelosuppression which comprises treating patients with therapeutically effective doses of stem cell factor.

39. A DNA sequence coding for an analog of human stem cell factor selected from the group consisting of:

a) [Met^{-1}] stem cell factor; and

b) stem cell factor wherein one or more cysteines are replaced by alanine or serine.

40. A polypeptide product of the expression in a procaryotic or eukaryotic host cell of a DNA sequence according to Claim 39.

41. A pharmaceutical composition comprising recombinant stem cell factor having the human amino acid sequence, and a pharmaceutically acceptable diluent, adjuvant or carrier.

42. An antibody specifically binding stem cell factor.

43. An antibody as in Claim 42 wherein said antibody is a monoclonal antibody.

44. A process for the efficient recovery of stem cell factor from SCF containing material, the method comprising the step of subjecting the SCF containing material to ion exchange chromatographic separation.

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45. A process as in Claim 44 wherein said ion exchange chromatographic separation is anion exchange chromatographic separation.

46. A process as in Claim 44 further comprising the step of reverse phase liquid chromatographic separation.

47. A process for the efficient recovery of stem cell factor from SCF containing material, the method comprising the step of subjecting the SCF containing material to reverse phase liquid chromatographic separation.

48. A polypeptide having the hematopoietic biological activity of naturally occurring stem cell factor, said polypeptide having an amino acid sequence set forth in Figure 15C, Figure 42 or Figure 44 or any allelic variants, derivatives, deletion analogs, substitution analogs, or addition analogs thereof, and characterized by being the product of procaryotic or eucaryotic expression of an exogenous DNA sequence.

49. A polypeptide as in Claim 48 selected from the group consisting of:

SCF^{1-148}, SCF^{1-162}, SCF^{1-164}, SCF^{1-165}, SCF^{1-183} (Figure 15C); SCF^{1-185}, SCF^{1-188}, SCF^{1-189}, and SCF^{1-248} (Figure 42); and SCF^{1-157}, SCF^{1-160}, SCF^{1-161} and SCF^{1-220} (Figure 44).

50. A biologically active composition comprising the polypeptide of Claim 1 covalently attached to a water-soluble polymer.

51. A composition as in Claim 50 wherein said polymer is selected from the group consisting of polyethylene glycol or copolymers of polyethylene glycol and polypropylene glycol, and said polymer is unsubstituted or substituted at one end with an alkyl group.

52. The composition of Claim 50 wherein the polypeptide is [Met^{-1}] SCF^{1-164}.

53. The composition of Claim 50 wherein said polymer has an average molecular weight of about 1,000 to 100,000 daltons.

54. The composition of Claim 50 wherein said polymer has an average molecular weight of about 4,000 to 40,000 daltons.

55. The composition of Claim 50 wherein said polymer is an unsubstituted polyethylene glycol or a monomethoxy polyethylene glycol.

56. The composition of Claim 50 wherein said polymer is attached to said polypeptide via reaction with an active ester of a carboxylic acid or carbonate derivative of said polymer.

57. The composition of Claim 50 wherein one or more amino groups of said protein are conjugated to said polymer by reaction with a N-hydroxysuccinimide, p-nitrophenol or 1-hydroxy-2-nitro-benzene-4-sulfonate ester of the polymer.

58. The composition of Claim 50 wherein one or more free cysteine sulfhydryl groups are conjugated to said polymer via reaction with a maleimido or haloacetyl derivative of the polymer.

59. The composition of Claim 50 wherein the polypeptide is glycosylated and the polymer is attached by reaction of an amino, hydrazine or hydrazide derivative of the polymer with one or more aldehyde groups generated by oxidation of the carbohydrate moieties.

60. A method for preparing a biologically active polymer-polypeptide adduct which comprises reacting the polypeptide of Claim 50 with a water-soluble polymer under conditions permitting the covalent attachment of the polymer to said polypeptide, and recovering the adduct so produced.

61. A method of treating acquired immune deficiency in a human comprising administering a therapeutically effective amount of the polypeptide according to Claim 1.

62. A method of treating neoplasia in a mammal comprising administering a therapeutically effective amount of the polypeptide according to Claim 1.

63. A method as in Claim 62 wherein before said administering step is the step of administering chemotherapy or irradiation to said mammal.

64. A method of transfecting early hematopoietic progenitor cells with a gene comprising:

(i) culturing early hematopoietic progenitor cells with SCF; and

(ii) transfecting the cultured cells of step (i) with a gene.

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65. A method of transferring a gene to a mammal comprising the steps of:

(i) culturing early hematopoietic progenitor cells with SCF;

(ii) transfecting the cultured cells of step (i) with a gene; and

(iii) administering the transfected cells to said mammal.

66. A method of treating nerve damage in a mammal comprising administering a therapeutically effective amount of the polypeptide according to Claim 1.

67. A method of treating infertility in a mammal comprising administering a therapeutically effective amount of the polypeptide according to Claim 1.

68. A method of treating intestinal damage in a mammal comprising administering a therapeutically effective amount of the polypeptide according to Claim 1.

69. A method of treating myeloproliferative disorder in a mammal comprising administering a therapeutically effective amount of the polypeptide according to Claim 1 conjugated to a toxin.

70. A polypeptide as in Claim 48 selected from the group consisting of:

$[Met^{-1}]SCF^{1-148}$, $[Met^{-1}]SCF^{1-162}$, $[Met^{-1}]SCF^{1-164}$, $[Met^{-1}]SCF^{1-165}$, $[Met^{-1}]SCF^{1-183}$ (Figure 15C); $[Met^{-1}]SCF^{1-185}$, $[Met^{-1}]SCF^{1-188}$, $[Met^{-1}]SCF^{1-189}$, and $[Met^{-1}]SCF^{1-248}$ (Figure 42); and $[Met^{-1}]SCF^{1-157}$, $[Met^{-1}]SCF^{1-160}$, $[Met^{-1}]SCF^{1-161}$ and $[Met^{-1}]SCF^{1-220}$ (Figure 44).

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