METHOD OF PRODUCING RECOMBINANT TAT-HOXB4H PROTEIN FOR USE AS A STIMULANT OF HEMATOPOIESIS *IN VIVO*

Field of the Invention

[001] The present invention relates to a new and nonobvious method of producing the C-terminal histidine tagged TAT-HOXB4 fusion protein (TAT-HOXB4H) containing at least 6 histidine residues at the C-terminus. The method of production provides unexpected benefits of increased stability and yield, which allows for successful *in vivo* administration of this protein.

Background of the Invention

[002] The growing interest in regenerative medicine has fueled the search for organ-specific stem or self-renewing cells. The best studied population of self-renewing cells is the hematopoietic stem cells (HSCs) as they are innovative options for treatment of diseases from cancer to metabolic disease to immunodeficiencies.

[003] The process of blood cell formation whereby red and white blood cells are replaced through the division of HSCs located in the bone marrow is called hematopoiesis. The HSCs have the key properties of being able to self-renew and to differentiate into mature cells of both lymphoid and myeloid lineages. However, the genetic mechanisms responsible for the control of self-renewal and differentiation outcomes of HSC divisions remain largely unknown.

[004] Currently, transplantation of human HSCs from adult bone marrow, mobilized peripheral blood, and umbilical cord blood (UCB) has been used clinically to treat hematopoietic cancers (leukemias and lymphomas) and to aid immune system recovery from high-dose chemotherapy of non-hematopoietic cancers. However, efficient transplantation requires substantial amount of HSCs from different sources and may require expansion.

[005] HSCs can be originated from bone marrow, peripheral blood and UCB. The extraction of bone marrow cells requires surgery and painful procedure, and therefore becomes less favorable approach. Using peripheral blood cells is also a problem because the difficulty of obtaining qualified HSCs from the hematopoiesis compromised patient who suffer from illness or chemotherapy. UCBs are relatively easier obtained and the quality of HSCs is much higher, however, the number of HSCs obtained from this approach is still limited. Cell number from each extraction is sufficient for a child, but may be insufficient for an adult. To overcome this potential problem, a new approach that facilitates HSC proliferation in vitro by intervening stem cell self-renewal process is indeed necessary for HSC transplantation.

[006] It has been indicated that transcription factors play a critical role in the regulation of gene expression and the differentiation in stem cells (Orkin, S. H. *Nature Reviews Genetics* 1, 57-64, 2000). Transcription factor switches various cellular processes through binding to specific gene target, and this regulation also depends on its cellular concentration. A group of transcription factors called DNA binding homeobox (HOX) was previously found to play a major

role in embryogenesis. Recently, HOX family is also found to be involved in the development of HSCs (Buske, C. et. al., *J. Hematol.* 71, 301-308, 2000). The regulation of HSC self-renewal by HOX transcription factor was studied by Dr. Guy Sauvageau from the University of Montreal. Sauvageau's group showed that homeobox gene HOXB4 is critical in the regulation of HSC self-renewal for its ability of maintaining HSC population in bone marrow. HOX genes expressed in blood cells was first observed in human and mouse cell lines. Some types of HOX genes are expressed ubiquitously in various cell types, while others are specifically expressed in certain type of cells or certain time points during the development. For example, eight members of human HOXB cluster are expressed in early stage of erythrocyte development. However, HOXB genes such as HOXB4 and HOXB7 are also expressed in T cells and B cells. Sauvageau's group confirmed that nine HOXA, eight HOXB and four HOXC genes are expressed in $CD34^+$ bone marrow cells. Among these $CD34^+$ bone marrow cells, HOXB2, HOXB9 and HOXA10 are most enriched in erythrocyte progenitor cells. However, no HOX genes are expressed in $CD34^-$ cells. Human homeobox B4 (HOXB4) gene was recently demonstrated to effectively expand HSCs in a retroviral or recombinant protein form. Recombinant TAT-HOXB4 proteins were used to expand stem cells in the laboratory scale without the risk of retroviral insertion or co-culture with bone marrow stromal cells (*See* Krosl, J. et al., *Nature Medicine* 9, 1428-1432, 2003). Therefore, HOXB4 protein is regularly used as a stimulant to promote HSCs expansion *in vitro* (Figure 1).

[007] Recent evidence indicated that by adding a TAT protein sequence tag at the N-terminus of HOXB4, exogenous HOXB4 can be delivered into the cell. This TAT sequence directs the transportation of HOXB4 from extracellular side into intracellular side. Upon entering the cytosol, HOXB4 can be refolded into its native conformation by chaperon HSP90. TAT-HOXB4 is able to promote HSC proliferation to 2-6 fold (Amsellem, S. et. al., *Nature Medicine* 9, 1423-1427, 2003; Krosl, J. et. al., *Nature Medicine* 9, 1428-1432, 2003). However, the yield of recombinant TAT-HOXB4 protein from *E. coli* by using regular purification procedure is too low.

[008] In an effort to increase the yield of the recombinant TAT-HOXB4 protein, a method of making a TAT-HOXB4H protein with additional six histidine residues tagged at the C-terminus was developed which resulted in 3-4 fold yield compared to that of the original protein after purification. The resultant recombinant protein (TAT-HOXB4H) contains 6 histidine residues at the C-terminus. This method was described in detail in the PCT application PCT/CN2006/000646.

[009] It was shown that the recombinant TAT-HOXB4H protein can be used to expand human peripheral blood or UCB stem cells and the expanded stem cells still possess their pluripotency. Furthermore, the stem cells treated with the recombinant TAT-HOXB4H protein incorporated into the bone marrow of nonobese diabetic-severe combined immunodeficiency (NOD-SCID) mice and human leukocytes were detected in peripheral white blood cells, indicating immune and hematopoiesis reconstitution in the mice.

[010] However, recombinant TAT-HOXB4H proteins have never been used before as a stimulator of hematopoiesis *in vivo*, specifically, to enhance hematopoietic reconstitution, expansion, bone marrow re-population and to increase the number of peripheral circulating stem cells, particularly after chemotherapy or irradiation. Krosl et al. (2003) and Amsellem et al. (2003) were not able to obtain large amounts of highly stable HOXB4 protein to be used in clinical studies to expand HSCs. In the present invention, the total amount of TAT-HOXB4H protein obtained after purification generally ranges from 6-10 mg from a 1 liter culture, while the total amount of TAT-HOXB4 protein obtained after purification from a 1 liter culture using prior art methods generally ranges from 1-2 mg. The pTAT-HA-HOXB4 plasmid used to express the TAT-HOXB4 protein using prior art methods was a gift from Dr. Guy Sauvageau, University of Montreal, Canada. The method of purifying the TAT-HOXB4H protein using the present invention clearly indicates the increased yield of protein necessary for the *in vivo* administration. Krosl et al. (2003) also reported that most of their TAT-HOXB4 protein was lost after 4 h of incubation in medium with serum. The present invention shows a significantly high stability of TAT-HOXB4H protein even after 4 weeks, which is a key factor for the use of TAT-HOXB4 protein in clinical studies.

Summary of the Invention

[011] The present invention is based on the new and nonobvious method of producing the TAT-HOXB4H protein with high yield and stability, and on the finding that recombinant TAT-HOXB4H protein, when administered to a

subject in need thereof, increase number of HSCs in both the bone marrow and peripheral blood *in vivo*.

[012] One aspect of the invention relates to a method of producing a TAT-HOXB4H protein. The method comprises: (a) providing a host cell comprising a vector encoding the protein; (b) expressing the protein in the host cell; (c) collecting an impure solution of the expressed protein; (d) purifying the protein from the solution by: (i) applying the solution to a HisTrap column; (ii) washing the HisTrap column; (iii) eluting the partially purified protein from the HisTrap column to form a partially purified protein solution; (iv) applying the partially purified protein solution to a MonoSP column; (v) washing the MonoSP column; (vi) eluting the purified protein from the MonoSP column in denatured form; (e) refolding the eluted denatured protein using hydrophobic compounds by (i) combining the eluted denatured protein and a solution of hydrophobic compounds to form a solution of protein and hydrophobic compounds; (ii) desalting the solution of protein and hydrophobic compounds to obtain a desalted protein and hydrophobic compound solution; (iii) removing the hydrophobic compounds from the desalted protein solution using ultrafiltration.

[013] One aspect of the invention relates to a method for enhancing the mobilization of HSCs from bone marrow to peripheral blood. The method comprises: a) administering an effective amount of a TAT-HOXB4H protein produced by the methods described herein to a subject in need thereof , and b) allowing the TAT-HOXB4H protein to increase the absolute number of

hematopoietic stem cells in the bone marrow of the subject thereby enhancing the mobilization of hematopoietic stem cells to the peripheral blood of the subject.

[014] Another aspect of the invention relates to a method for improving the recovery time of a patient having undergone HSC transplantation, irradiation or chemotherapy. The method comprises: a) administering an effective amount of a TAT-HOXB4H protein produced by the methods described herein to a subject in need thereof, and b) allowing the TAT-HOXB4H protein to increase the absolute number of HSCs to the bone marrow of the subject.

[015] One aspect of the invention relates to a pharmaceutical composition for mobilization of HSCs from bone marrow to peripheral blood in a subject in need thereof. The pharmaceutical composition of the invention includes an effective amount of a TAT-HOXB4H protein produced by the methods described herein sufficient to increase the absolute number of HSCs in the bone marrow of the subject thereby enhancing the mobilization of HSCs to the peripheral blood of the subject.

[016] The pharmaceutical composition of the invention may be administered to a patient having undergone autologous HSC transplantation for improving the recovery time after HSC transplantation.

[017] The pharmaceutical composition of the invention may be administered to a granulocyte-colony stimulating factor (G-CSF)-insensitive patient as a substitute for G-CSF for mobilization of HSCs to peripheral blood.

[018] The pharmaceutical composition of the invention may be administered to a HSC donor thereby allowing a sufficient amount of HSCs to be

collected for transplantation in a much less invasive procedure from the peripheral blood rather than the bone marrow of said donor.

[019] Another aspect of the invention relates to treatment of diseases caused by inherited HSC deficiency by systemically administering an effective amount of a recombinant TAT-HOXB4H protein produced by the methods described herein or of a pharmaceutical composition comprising the same to a subject suffering from the diseases. The administered recombinant TAT-HOXB4H protein thereby increases the absolute number of HSCs in the bone marrow of the subject.

[020] A further aspect of the invention relates to a method for improving the recovery time after HSC transplantation by systemically administering an effective amount of a recombinant TAT-HOXB4H protein produced by the methods described herein or of a pharmaceutical composition comprising the same to a subject in need thereof.

[021] A still further aspect of the invention relates to a method for enhancing HSC recovery of a patient receiving irradiation or chemotherapy by systemically administering an effective amount of a recombinant TAT-HOXB4H protein produced by the methods described herein or of a pharmaceutical composition comprising the same to a subject in need thereof.

Brief Description of the Figures

[022] The foregoing aspects and advantages of this invention may become apparent from the following detailed description with reference to the accompanying drawings in which:

[023] Figure 1 shows a schematic representation of mobilization of HSCs from CD34 cells to peripheral blood (PB) by *in vivo* expansion.

[024] Figure 2 shows schematic representation of construction and cloning of pTAT-HOXB4H in modified pET21b vector.

[025] Figure 3 represents the DNA sequence of pTAT-HOXB4H. The additional 6 histidine residues introduced at the N- and C-termini in pTAT-HOXB4H are underlined and TAT is highlighted.

[026] Figure 4 shows the protein sequence of TAT-HOXB4H protein.

[027] Figure 5 shows 10% SDS-polyacrylamide gel (1.5 mm) demonstration of the purification of TAT-HOXB4H protein. The SDS-polyacrylamide gel was stained with coomassie blue. Lane 1, molecular weight markers (M), 0.3 μg protein; lane 2, cell lysate from uninduced BL21(DE3)pLysS cells expressing TAT-HOXB4H protein, 1 μg protein; lane 3, cell lysate from induced BL21(DE3)pLysS cells expressing TAT-HOXB4H protein with 1 mM IPTG, 1 μg protein; lane 4, purified TAT-HOXB4H, 0.7 mg protein; lane 5, purified TAT-HOXB4 (0.2 mg protein). The pTAT-HA-HOXB4 plasmid used to express the TAT-HOXB4 protein (lane 5) was a gift from Dr. Guy Sauvageau,

University of Montreal, Canada. Equal volume of fractions, that were collected from MonoSP column, were loaded in the lanes 4 and 5.

[028] Figure 6 shows SDS-polyacrylamide gel analysis of the stability of purified TAT-HOXB4H protein stored in PBS for 0 hours (A) and 16 hours (B) at 4 °C M represents molecular weight markers, 0 represents 0 hours incubation at 4 °C, 16 represents 16 hour incubation at 4 °C.

[029] Figure 7 shows the stability of TAT-HOXB4H protein stored at 4 °C and -20 °C in PBS and storage buffer, IMDM, analyzed by 10% SDS-polyacrylamide followed by coomassie staining. Arrows indicate TAT-HOXB4H protein bands.

[030] Figure 8A shows the stimulatory effect of G-CSF on the number of $CD34^+$ stem cells in bone marrow of mice analyzed by a flow cytometer.

[031] Figure 8B shows the effect of PBS on the number of $CD34^+$ stem cells in bone marrow of mice analyzed by a flow cytometer.

[032] Figure 8C shows the stimulatory effect of TAT-HOXB4H on the number of $CD34^+$ stem cells in bone marrow of mice analyzed by a flow cytometer.

[033] Figure 9A shows the stimulatory effect of G-CSF on the number of $CD34^+$ stem cells in bone marrow of rhesus monkey analyzed by a flow cytometer.

[034] Figure 9B shows the stimulatory effect of TAT-HOXB4H together with G-CSF on the number of $CD34^+$ stem cells in bone marrow of rhesus monkey analyzed by a flow cytometer.

[035] Figure 9C shows the stimulatory effect of TAT-HOXB4H on the number of $CD34^+$ stem cells in bone marrow of rhesus monkey analyzed by a flow cytometer.

[036] Figure 9D shows the effect of PBS on the number of $CD34^+$ stem cells in bone marrow of rhesus monkey analyzed by a flow cytometer.

[037] Figure 10 shows effect of TAT-HOXB4H protein on hematopoietic recovery in NOD-SCID mice.

[038] Figure 11 shows effect of TAT-HOXB4H protein on hematopoietic recovery in Balb/c mice after cisplatin chemotherapy.

Detailed Description

I. The TAT-HOXB4H Protein

[039] The present invention relates to a new and nonobvious method of producing the TAT-HOXB4H protein, providing unexpected benefits of increased stability and yield, which allows for *in vivo* administration of this protein. The TAT-HOXB4H protein is a construct comprising three elements: TAT, HOXB4, and a histidine tag. HOXB4 is a member of the HOX family of transcription factors and promotes HSC expansion. TAT allows the HOXB4 moiety to be transported into the cell. The histidine tag allows for initial increased yield from recombinant expression sources, although the method of production further increases the yield of the protein. pTAT-HOXB4H has been constructed as shown in Figure 2, and the DNA sequence is shown in Figure 3.

The recombinant TAT-HOXB4H protein refers to a TAT-HOXB4 fusion protein with additional six histidine residues tagged at the C-terminus (Figure 4)

[040] Unless otherwise indicated, a protein's amino acid sequence (*i.e.*, its "primary structure" or "primary sequence") may be written from amino-terminus to carboxy-terminus. In non-biological systems (for example, those employing solid state synthesis), the primary structure of a protein (which also includes disulfide (cysteine) bond locations) can be determined by the user.

[041] A "deletion" refers to a change in an amino acid or nucleotide sequence due to the absence of one or more amino acid residues or nucleotides. The terms "insertion" or "addition" refer to changes in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid residues or nucleotides, respectively, to a molecule or representation thereof, as compared to a reference sequence, for example, the sequence found in the naturally occurring molecule. A "substitution" refers to the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively.

[042] Sequences similar or homologous (e.g., at least about 85% sequence identity) to the sequences disclosed herein are also part of this application. In some embodiment, the sequence identity can be about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher. Alternatively, substantial identity exists when the nucleic acid segments may hybridize under selective hybridization conditions (e.g., highly stringent hybridization conditions), to the complement of the strand. The nucleic acids may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form.

[043] Calculations of "homology" or "sequence identity" between two sequences (the terms are used interchangeably herein) are performed as follows. The sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, 90%, 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

[044] The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch ((1970) *J. Mol.*

Biol. 48:444-453) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. A particularly preferred set of parameters (and the one that may be used if the practitioner is uncertain about what parameters may be applied to determine if a molecule is within a sequence identity or homology limitation of the invention) are a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frame shift gap penalty of 5. The percent identity between two amino acid or nucleotide sequences can also be determined using the algorithm of E. Meyers and W. Miller ((1989) CABIOS, 4:11-17) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

II. Methods of Making the Recombinant TAT-HOXB4H Protein

A. Cloning and Expression

[045] Systems for cloning and expressing proteins in a variety of host cells are known in the art. Cells suitable for producing proteins are described in, for example, Fernandez et al. (1999) Gene Expression Systems, Academic Press, eds. In brief, suitable host cells include mammalian cells, insect cells, plant

cells, yeast cells, or prokaryotic cells, e.g., *E. coli*. Mammalian cells available in the art for heterologous protein expression include lymphocytic cell lines (e.g., NSO), HEK293 cells, Chinese hamster ovary (CHO) cells, COS cells, HeLa cells, baby hamster kidney cells, oocyte cells, and cells from a transgenic animal, e.g., mammary epithelial cell. Suitable vectors may be chosen or constructed to contain appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes, and other sequences. The vectors may also contain a plasmid or viral backbone. For details, see Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press (1989). Many established techniques used with vectors, including the manipulation, preparation, mutagenesis, sequencing, and transfection of DNA, are described in Current Protocols in Molecular Biology, Second Edition, Ausubel et al. eds., John Wiley & Sons (1992).

[046] A further aspect of the disclosure provides a method of introducing the nucleic acid into a host cell. For eukaryotic cells, suitable transfection techniques may include calcium phosphate, DEAE-Dextran, electroporation, liposome-mediated transfection, and transduction using retrovirus or other viruses, e.g., vaccinia or baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation, and transfection using bacteriophage. DNA introduction may be followed by a selection method (e.g., drug resistance) to select cells that contain the nucleic acid.

B. **Purification and Refolding**

[047] TAT-HOXB4H protein can be initially isolated from the recombinant host cell by any appropriate means known in the art. For example, the protein can be removed from cell supernatant, if the protein is capable of being secreted, or the protein can be removed from a cell lysate.

[048] TAT-HOXB4H protein can be purified using the chromatographic methods comprising: (a) applying the cell lysate or cell supernatant, if the protein is being secreted into the solution, to a HisTrap column; b) washing the HisTrap column with a buffer; (c) eluting the partially purified protein from the HisTrap column (d) applying the partially purified protein obtained from HisTrap column to a MonoSP column; (e) washing the MonoSP column with a buffer; (f) eluting the purified TAT-HOXB4H protein from the MonoSP column.

[049] Cell lysate or cell supernatant, if the protein is capable of being secreted into the solution, can be cleared by centrifugation at 20,000 x g for 30 min at 4 °C, the supernatant adjusted to 10 mM imidazole and loaded on the HisTrap chelating columns (Amersham Pharmacia). The column can be washed with 8 M urea, 20 mM HEPES, 0.5 mM DTT, 10 mM NaCl, pH 8.0 and 10 mM imidazole to remove unbound proteins. Partially pure TAT-HOXB4 protein can be eluted from the HisTrap column with high concentration of imidazole and salt.

[050] For further purification, partially purified protein obtained from HisTrap column can be applied to MonoSP column (Amersham Pharmacia). The column can be washed with 4 M urea, 20 mM HEPES, 50 mM NaCl, pH 6.5 to remove unbound proteins. Bound TAT-HOXB4H can be eluted with high salt.

The purified TAT-HOXB4H protein collected from this purification procedure is in the denatured form.

[051] Further, the denatured TAT-HOXB4H protein eluted from the MonoSP column can be refolded using hydrophobic compounds by (i) combining the eluted denatured protein and a solution of hydrophobic compounds to form a solution of protein and hydrophobic compounds; (ii) desalting the solution of protein and hydrophobic compounds to obtain a desalted protein and hydrophobic compound solution; and (iii) removing the hydrophobic compounds from the desalted protein solution using ultrafiltration.

[052] As used in the present invention, the term "hydrophobic compounds" refer to any hydrophobic compounds capable of protecting the desired protein from forming insoluble aggregates during the denaturing salt-removing step. Hydrophobic compounds suitable for use in the present invention are described in Oganesyan et al., *Pharmagenomics* (2004) 71, 22-26. Suitable hydrophobic compounds include, but are not limited to, Triton X-100, tween-20 or polybenzene compounds. Ultrafiltration or buffer exchange can be carried out by a centricon or stir-cell. The conditions for ultrafiltration or buffer exchange may vary, as recognized by those skilled in the art, depending on the types of the desired protein.

[053] In one embodiment of the present invention, the hydrophobic compound in the desalted solution containing denatured HOXB4H protein is removed by 5-10 times of buffer exchange (each performed by centrifugation at 1000-2500 x g for 10 min) with solution containing low to high concentrations of

large hydrophobic compounds such as beta-cyclodextrin whereby the denatured HOXB4H protein is refolded into native form thereof.

[054] In one embodiment, purified TAT-HOXB4H protein can be stored in commercially available IMDM (HyClone) medium (storage buffer 1) at 4 °C or -20 °C.

[055] In another embodiment, purified TAT-HOXB4H protein can be stored in commercially available DMEM (HyClone) medium (storage buffer 2) at 4 °C or -20 °C.

[056] In one embodiment, His tag at the C-terminus of TAT-HOXB4H may be removed before *in vivo* administration.

[057] In another embodiment His tag at the N-terminus of TAT-HOXB4H can be removed before *in vivo* administration.

[058] In another embodiment both His tags at the N- and C- termini can be removed before *in vivo* administration.

C. Preparation of a Pharmaceutical Composition

[059] TAT-HOXB4H may be used as a pharmaceutical composition when combined with a pharmaceutically acceptable carrier. Such a composition may contain, in addition to the TAT-HOXB4H protein and carrier, various diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials well known in the art. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). The characteristics of the carrier may depend on the route of administration.

[060] It is especially advantageous to formulate compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

[061] Typical routes of administration include, without limitation, oral, topical, parenteral (*e.g.*, sublingually or buccally), sublingual, rectal, vaginal, and intranasal. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intrasternal, intracavernous, intrathecal, intrameatal, intraurethral injection or infusion techniques. The pharmaceutical composition is formulated so as to allow the active ingredients contained therein to be bioavailable upon administration of the composition to a patient. Compositions that may be administered to a patient take the form of one or more dosage units, where for example, a tablet may be a single dosage unit, and a container of one or more compounds of the invention in aerosol form may hold a plurality of dosage units.

[062] When a therapeutically effective amount of a TAT-HOXB4H protein is administered orally, the binding agent may be in the form of a tablet,

capsule, powder, solution or elixir. When administered in tablet form, the pharmaceutical composition of the invention may additionally contain a solid carrier such as a gelatin or an adjuvant. The tablet, capsule, and powder contain from about 5 to 95% binding agent, and preferably from about 25 to 90% binding agent. Examples are sucrose, kaolin, glycerin, starch dextrins, sodium alginate, carboxymethylcellulose and ethyl cellulose. Coloring and/or flavoring agents may be present. A coating shell may be employed. When administered in liquid form, a liquid carrier such as water, petroleum, oils of animal or plant origin such as peanut oil, mineral oil, soybean oil, or sesame oil, or synthetic oils may be added. The liquid form of the pharmaceutical composition may further contain physiological saline solution, dextrose or other saccharide solution, or glycols such as ethylene glycol, propylene glycol or polyethylene glycol. When administered in liquid form, the pharmaceutical composition contains from about 0.5 to 90% by weight of the binding agent, and preferably from about 1 to 50% the binding agent.

[063] When a therapeutically effective amount of TAT-HOXB4H protein is administered by intravenous, cutaneous or subcutaneous injection, binding agent may be in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such parenterally acceptable protein solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. In some embodiments, pharmaceutical composition for intravenous, cutaneous, or subcutaneous injection may contain, in addition to binding agent an isotonic vehicle such as Sodium Chloride Injection, Ringer's

Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additive known to those of skill in the art.

[064] In practicing the method of treatment or use of the present invention, a therapeutically effective amount of TAT-HOXB4H protein is administered to a subject, e.g., mammal (e.g., a human). As used herein, the term "therapeutically effective amount" means the total amount of each active component of the pharmaceutical composition or method that is sufficient to show a meaningful patient benefit, e.g., amelioration of symptoms of, healing of, or increase in rate of healing of such conditions. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

[065] The amount of TAT-HOXB4H protein in the pharmaceutical composition of the present invention may depend upon the nature and severity of the condition being treated, and on the nature of prior treatments that the patient has undergone, and patient's age and sex. Ultimately, the attending physician may decide the amount of active ingredient with which to treat each individual patient. Initially, the attending physician may administer low doses of active ingredient and observe the patient's response. Larger doses of active ingredient may be administered until the optimal therapeutic effect is obtained for the patient,

and at that point the dosage is not generally increased further. It is contemplated that the various pharmaceutical compositions used to practice the method of the present invention may contain about 1 μg to about 1 mg TAT-HOXB4H protein per kg body weight. Examples of dosage ranges that can be administered to a subject can be chosen from: 1 μg/kg to 1 mg/kg, 1 μg/kg to 0.5 mg/kg, 1 μg/kg to 0.1 mg/kg, 10 μg/kg to 0.5 mg/kg, 10 μg/kg to 0.1 mg/kg, 100 μg to 0.5 mg/kg, 250 μg/kg to 0.5 mg/kg. Further, examples of dosage ranges that can be chosen from: 50 μg to 100 mg, 100 μg to 50 mg, 500 μg to 50 mg, 1 mg to 50 mg. The duration of intravenous therapy using the pharmaceutical composition of the present invention may vary, depending on the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. In one embodiment, it is contemplated that the duration of each application of the TAT-HOXB4H protein may be in the range of 12 to 24 hours of continuous intravenous administration. In another embodiment, the duration of application of TAT-HOXB4H protein may last as long as patient's radiation or chemotherapy continues. TAT-HOXB4 protein may be administered in the range of 10-100 μg/kg intravenously, twice a day for 4.5 to 5 days. One cycle of the treatment may be enough to expand HSCs *in vivo*. Ultimately the attending physician may decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

[066] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Data obtained from the cell culture assays and animal studies can be used in evaluating a range of dosage for use in humans. The dosage of such compounds may lie within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The therapeutically effective dose of TAT-HOXB4H can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of the test protein which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Levels in plasma may be measured, for example, by high performance liquid chromatography. The effects of any particular dosage can be monitored by a suitable bioassay. Examples of suitable bioassays include, but not limited to, measuring $CD34^+$ stem cells in the mononuclear cell by using fluorescent probe (e.g., FITC) tagged antibodies to CD34+ stem cells and measuring the percentage of LY5 cells in the peripheral blood or bone marrow HSCs by flow cytometry. The polynucleotide and proteins of the present

invention are expected to exhibit one or more of the uses or biological activities (including those associated with assays cited herein) identified below. Uses or activities described for proteins of the present invention may be provided by administration or use of such proteins or by administration or use of polynucleotides encoding such proteins (such as, for example, in gene therapies or vectors suitable for introduction of DNA).

III. Methods of Stimulating Hematopoiesis *In Vivo*

A. Patients in Need of Treatment

[067] The pharmaceutical compositions of the invention may be used to treat diseases which include, but are not limited to, autoimmune disorders, immunodeficiency disorders, and hematological disorders. Additionally, pharmaceutical compositions of the invention may be used to improve the recovery time after HSC transplantation.

[068] The pharmaceutical composition of the invention include, but are not limited to, the treatment of patient suffering from or susceptible to lymphomas, leukemias, Hodgkin's disease and myeloproliferative disorders. Additionally, inherited diseases caused by HSC deficiency and aplastic anemia may be treated by the pharmaceutical composition of the present invention.

[069] Further the pharmaceutical composition of the present invention may be employed to the HSC donors and G-CSF-insensitive patients.

[070] In one embodiment of the invention, TAT-HOXB4H is the only active agent administered for mobilization of HSCs, and fluorouracil (5-FU) is not

administered to the donor, either as pretreatment or a combination therapy scheme.

[071] Additional diseases or conditions associated with increased cell survival, that may be treated by the pharmaceutical composition of the invention include, but are not limited to, progression, and/or metastases of malignancies and related disorders such as leukemia (including acute leukemias (for example, acute lymphocytic leukemia, acute myelocytic leukemia (including myeloblastic, promyelocytic, myelomonocytic, monocytic, and erythroleukemia)) and chronic leukemias (for example, chronic myelocytic (granulocytic) leukemia and chronic lymphocytic leukemia)), myelodysplastic syndrome polycythemia vera, lymphomas (for example, Hodgkin's disease and non-Hodgkin's disease), multiple myeloma, Waldenström's macroglobulinemia, and solid tumors including, but not limited to, sarcomas and carcinomas such as fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder

carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, melanoma, neuroblastoma, and retinoblastoma.

[072] The present invention is not limited to the particular methodology, protocols, cell lines, animal species or genera, and reagents described below. The terminology used to describe particular embodiments is not intended to limit the scope of the present invention, which may be limited only by the appended claims. As used herein, the singular forms "a," "and," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" is a reference to one or more cells and includes equivalents thereof known to those skilled in the art.

[073] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, devices, and materials are now described. All publications and patents mentioned herein are hereby incorporated herein by reference for the purpose of describing and disclosing, for example, the constructs and methodologies that are described in the publications which might be used in connection with the presently described invention. The publications discussed above and throughout the text are provided solely for their disclosure prior to the effective date of the present application. Nothing herein is

to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

Definitions

[074] A "stem cell" is a pluripotent or multipotent cell with the ability to self-renew, to remain undifferentiated, and to become differentiated. Stem cells can divide without limit, at least for the lifetime of the animal in which they naturally reside. Stem cells are not terminally differentiated, i.e., they are not at the end of a pathway of differentiation. When a stem cell divides, each daughter cell can either remain a stem cell or it can embark on a course that leads to terminal differentiation. A "chimeric" stem cell is a stem cell with a portion of its DNA belonging to a heterologous organism.

[075] A "hematopoeitic" cell is a cell involved in the process of hematopoeisis, i.e., the process of forming mature blood cells from precursor cells. In the adult, hematopoeisis takes place in the bone marrow. Earlier in development, hematopoeisis takes place at different sites during different stages of development; primitive blood cells arise in the yolk sac, and later, blood cells are formed in the liver, spleen, and bone marrow. Hematopoeisis undergoes complex regulation, including regulation by hormones, e.g., erythropoietin; growth factors, e.g., colony stimulating factors; and cytokines, e.g., interleukins.

[076] The term "vector", as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another

type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

[077] "Transformation," as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective of the method used for insertion: for example, transformation by direct uptake, transfection, infection, and the like. For particular methods of transfection, see further below. The exogenous polynucleotide may be maintained as a nonintegrated vector, for example, an episome, or alternatively, may be integrated into the host genome.

[078] In general, the term "protein" refers to any polymer of two or more individual amino acids (whether or not naturally occurring) linked via peptide

bonds, as occur when the carboxyl carbon atom of the carboxylic acid group bonded to the α-carbon of one amino acid (or amino acid residue) becomes covalently bound to the amino nitrogen atom of the amino group bonded to the α-carbon of an adjacent amino acid. These peptide bond linkages, and the atoms comprising them (*i.e.*, α-carbon atoms, carboxyl carbon atoms (and their substituent oxygen atoms), and amino nitrogen atoms (and their substituent hydrogen atoms)) form the "polypeptide backbone" of the protein. In addition, as used herein, the term "protein" is understood to include the terms "polypeptide" and "peptide" (which, at times, may be used interchangeably herein). Similarly, protein fragments, analogs, derivatives, and variants are may be referred to herein as "proteins," and shall be deemed to be a "protein" unless otherwise indicated. The term "fragment" of a protein refers to a polypeptide comprising fewer than all of the amino acid residues of the protein. As may be appreciated, a "fragment" of a protein may be a form of the protein truncated at the amino terminus, the carboxy terminus, and/or internally (such as by natural splicing), and may also be variant and/or derivative. A "domain" of a protein is also a fragment, and comprises the amino acid residues of the protein required to confer biochemical activity corresponding to naturally occurring protein.

[079] "Recombinant" as used herein to describe a nucleic acid molecule means a polynucleotide of genomic, cDNA, viral, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation is not associated with all or a portion of the polynucleotide with which it is associated in nature. The

term "recombinant" as used with respect to a protein or polypeptide means a polypeptide produced by expression of a recombinant polynucleotide.

[080] An "isolated," "purified," "substantially isolated," or "substantially pure" molecule (such as a polypeptide or polynucleotide) is one that has been manipulated to exist in a higher concentration than in nature. For example, a subject protein is isolated, purified, substantially isolated, or substantially purified when at least 50%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more of non-subject-protein materials with which it is associated in nature have been removed. As used herein, an "isolated," "purified," "substantially isolated," or "substantially purified" molecule includes recombinant molecules.

[081] A "SCID mouse" is a mouse model for severe combined immunodeficiency syndrome (SCID), which causes severe defects in the development of the immune system. These mice are deficient in, or completely lack, both T and B lymphocytes. The SCID mutation appears to impair the recombination of antigen receptor genes, causing a lack of functional T and B lymphocytes. Other hematopoietic cell types appear to develop and function normally. SCID mice readily support normal lymphocyte differentiation and can be reconstituted with normal lymphocytes from syngeneic or allogeneic mice, or with human lymphocytes. These mice also support the growth of allogeneic and xenogeneic tumors. Therefore, SCID mice, which allow disseminated growth of a number of human tumors, particularly hematologic disorders and malignant melanoma, can be used to investigate malignancies.

[082] The terms "subject," "individual," "host," and "patient" are used interchangeably herein to refer to a living animal, including a human and a non-human animal. The subject may, for example, be an organism possessing immune cells capable of responding to antigenic stimulation, and stimulatory and inhibitory signal transduction through cell surface receptor binding. The subject may be a mammal, such as a human or non-human mammal, for example, dogs, cats, pigs, cows, sheep, goats, horses, rats, and mice. The term "subject" does not preclude individuals that are entirely normal with respect to a disease, or normal in all respects.

[083] The term "treatment" refers to a therapeutic or preventative measure. The treatment may be administered to a subject having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment.

[084] The term "therapeutically effective amount" means the amount of the subject compound that may elicit a desired response, for example, a biological or medical response of a tissue, system, animal, or human that is sought, for example, by a researcher, veterinarian, medical doctor, or other clinician.

Examples

[085] The following specific examples are to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. Without further elaboration, it is believed that one skilled in the art can, based on the description herein, utilize the present invention to its fullest extent.

Example 1: Construction of Plasmid pET21b-His-pTAT-HOXB4-His

(a) The modification of pET21b plasmid containing N- and C-terminal His Tag and TAT signal peptide.

[086] The expression vector pET21b containing N-terminal His tag and a TAT signal peptide was generated by inserting oligonucleotides 5'-TATGCACCACCACCACCACCACTACGGCCGCAAGAAACGCCGCCAGCGC CGCCGGCG-3' (sense) and 5'-CTAGCGGCGCTGGCGGCGTTTCTTGCGGCC GTAGTGGTGGTGGTGGTGGTGCA-3' (antisense) into pET21b plasmid. The C-terminal His-Tag is already present in the pET21b vector.

(b) Cloning of HOXB4 into modified pET21b expression vector

[087] A DNA fragment containing the open reading frame (ORF) of HOXB4 and additional 6 histidine coding sequence was obtained by PCR amplification using plasmid MGC54130 (GeneDiscovery, Taipei, Taiwan. Cat. No. 5533346) as the template, and the PCR-generated HOXB4 cDNA fragment was subcloned into the modified pET21b expression vector. Plasmid construction and nucleic acid sequences are shown in Figures 2 and 3.

Example 2: Expression of Recombinant TAT-HOXB4H Protein in *E. coli*

[088] The pET21b-His-TAT-HOXB4-His expression vector was transformed into *E. coli* strain BL21(DE3)pLysS (Novagen). The transformed cells were grown overnight at 37 °C. The overnight-grown cultures were diluted to an initial OD600 of 0.05. The cultures were then grown to an OD600 of 0.5 at 37 °C, induced with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) at 37 °C for 3 hr, with vigorous shaking.

Example 3: Purification of Recombinant TAT-HOXB4H protein

[089] Following induction, cells were harvested by centrifugation and resuspended in Buffer A (8 M Urea, 20 mM HEPES, 0.5 mM DTT and 100 mM NaCl, pH 8.0). The cell suspension was passed three times through a French press, and the cell lysate cleared by centrifugation at 20,000 x g for 30 min at 4 °C, adjusted the supernatant to 10 mM imidazole and loaded on the HisTrap chelating columns (Amersham Pharmacia). Bound proteins were eluted with 50, 100 and 250 mM imidazole in buffer A. TAT-HOXB4H containing fractions were loaded on a MonoSP column in Buffer B (4 M urea, 20 mM HEPES and 50 mM NaCl, pH 6.5), eluted with 1.5 M NaCl and 20 mM HEPES (pH 8.0).

Example 4: Renaturation of Recombinant TAT-HOXB4H Protein

[090] The TAT-HOXB4H protein in the eluted fractions was solubilized and denatured in a solution containing denaturing salt (e.g., guanidine hydrochloride) and then it was mixed with D-PBS-T buffer (0.1% Triton X-100 in 2x PBS). The ratio of the TAT-HOXB4H protein solution to D-PBS-T buffer was 1:4. The resultant mixture was added to a 10K centricon tube (50 ml or 15 ml)

pretreated with water (10 ml or 3 ml), and then centrifuged at 3000 rpm for 10 min. In this step, the denaturing salt is replaced by D-PBS-T buffer in which Triton X-100 is capable of binding with the hydrophobic region of the HOXB4H protein.

[091] This step of ultrafiltration or buffer exchange using 10K centricon was performed ten times with solution containing different concentrations of beta-cyclodextrin (two times of each 1 mM, 2 mM, 3 mM, 4 mM and 5 mM beta-cyclodextrin in storage buffer IMDM by centrifuging at 1000-2500 x g for 10 min. The remaining sample in the 10K centricon tube was collected and stored at -80℃.

[092] Homogeneity of the purified TAT-HOXB4H protein was analyzed by SDS-polyacrylamide gel followed by coomassie staining (figure 5). As shown in figure 5, TAT-HOXB4H purified from HisTrap and MonoSP resulted in 3-4 fold yield compared to that of the TAT-HOXB4 protein. The pTAT-HA-HOXB4 plasmid was a gift from Dr. Guy Sauvageau, University of Montreal, Canada. This plasmid was transformed into BL21(DE3)pLysS (Novagen) and the purification of TAT-HOXB4 protein was performed as described in Krosl et al, 2003.

Example 5: Stability of Recombinant TAT-HOXB4H protein

[093] The Stability of the TAT-HOXB4H was measured by SDS-polyacrylamide gel analysis. Upon storage full length TAT-HOXB4H can be degraded into 30 kD and 10 kD fragments. As shown in figure 6, TAT-HOXB4H protein produced by the method of this invention was stable even after 16 hours stored in PBS at 4 °C.

[094] Further, the stability of TAT-HOXB4H protein stored at 4 °C and -20 °C in PBS and storage buffer IMDM was analyzed by 10% SDS-polyacrylamide gel electrophoresis followed by coomassie staining. As shown in figure 7, when IMDM was used as a storage buffer, the stability of the TAT-HOXB4H protein was maintained even after 4 weeks.

Example 6: Effect of Recombinant TAT-HOXB4H Protein on Hematopoiesis in Wild Type Balb/c Mice

[095] Balb/c mice were used to investigate the possible effect of recombinant TAT-HOXB4H protein on mobilization of HSCs from bone marrow to peripheral blood. TAT-HOXB4H recombinant protein in phosphate buffered saline (PBS) was administered by subcutaneous injection four times per day for 4 days. To examine dose-responsiveness, experimental groups (n=21) received a dose ranged from 1 µg, 5 µg, 10 µg, 15 µg ... to 100 µg per kg BW per mouse. A separate group of control mice received phosphate buffered saline only, and another group of control mice were injected subcutaneously twice per day for 4 day with a dose of 5 µg per kg BW of G-CSF per mouse.

[096] Peripheral blood was harvested from all mice and analyzed by a flow cytometer to obtain the percentage of $CD34^+$ stem cells in mononuclear cell (MNC). The results are reported as the mean ± s.d. in Table 1.

Table 1

Group (Experimental/Control)	TAT-HOXB4H (µg/kg)	$CD34^+$/MNC (%)
1	1	0 ± 0.03
2	5	0.3 ± 0.05

Group (Experimental/Control)	TAT-HOXB4H (μg/kg)	$CD34^+$/MNC (%)
3	10	0.45 ± 0.03
4	15	0.42 ± 0.01
5	20	0.38 ± 0.05
6	25	0.41 ± 0.02
7	30	0.35 ± 0.21
8	35	0.33 ± 0.11
9	40	0.29 ± 0.16
10	45	0.46 ± 0.01
11	50	0.45 ± 0.02
12	55	0.42 ± 0.06
13	60	0.44 ± 0.02
14	65	0.41 ± 0.04
15	70	0.41 ± 0.03
16	75	0.49 ± 0.01
17	80	0.45 ± 0.04
18	85	0.46 ± 0.07
19	90	0.44 ± 0.02
20	95	0.41 ± 0.01
21	100	0.42 ± 0.05
Control (PBS)	0	0.002
Control (G-CSF)	0	0.5 ± 0.03

[097] The percentage of $CD34^+$/MNC in peripheral blood harvested from the treated mice is shown in Table 1. The TAT-HOXB4H treated

experimental groups 3-21 (received a dose of 10 μg or above per kg BW) showed substantially the same mobilization effect as G-CSF treated control group.

[098] Bone marrow from TAT-HOXB4H treated mice (Exp. Group 3 received a dose of 10 μg per kg BW), G-CSF treated mice, and PBS injected mice were further phenotyped using FITC-conjugated antibody to $CD34^+$ (Becton Dickinson) and analyzed by a flow cytometer. Bone marrow from mice treated with TAT-HOXB4H (Figure 5C) appears to be richer in $CD34^+$ stem cells than bone marrow from G-CSF (Figure 5A) and PBS (Figure 5B) injected mice. Therefore, these results indicate that injection of recombinant TAT-HOXB4H proteins results in increased number of HSCs in both the bone marrow and peripheral blood in mice.

Example 7: Effect of Recombinant TAT-HOXB4H Protein on Hematopoiesis in Rhesus Monkey

[099] Male adult rhesus monkeys were used to investigate the efficacy of recombinant TAT-HOXB4H protein in monkeys. Experimental group I (n=5) were injected intravenously with 10 μg per kg BW of recombinant TAT-HOXB4H protein four times per day for 4 days. Experimental group II (n=5) were injected intravenously with 10 μg per kg BW of TAT-HOXB4H four times per day and injected subcutaneously with 5 μg per kg BW of G-CSF for 4 days. Control group I received PBS only, and control group II were injected subcutaneously twice per day for 4 day with a dose of 5 μg per kg BW of G-CSF. Peripheral blood were harvested from all monkeys and analyzed by a flow

cytometer to obtain the percentage of $CD34^+$ stem cells in mononuclear cell (MNC). The results are presented in Table 2.

Table 2

Group (Experimental/Control)	$CD34^+$/MNC (%)
I. TAT-HOXB4H (10 μg/kg)	0.62
II. TAT-HOXB4H (10 μg/kg) + G-CSF (5 μg/kg)	0.38
Control 1 (PBS)	0.07
Control 2 (G-CSF, 5 μg/kg)	0.28

[0100] As shown in Table 2, monkeys (experimental group I) treated with TAT-HOXB4H only showed a significantly better mobilization effect than G-CSF treated monkeys (control group II). The monkeys treated with TAT-HOXB4H and G-CSF (experimental group II) showed a slightly better mobilization effect than G-CSF treated monkeys (control group II).

[0101] Bone marrow specimens from monkeys treated with TAT-HOXB4H, G-CSF or PBS were further phenotyped using FITC-conjugated antibody to $CD34^+$ (Becton Dickinson) and analyzed by a flow cytometer. Bone marrow specimens from monkeys treated with TAT-HOXB4H (Figure 6C) appears significantly richer in $CD34^+$ stem cells than bone marrow specimens from G-CSF (Figure 6A), TAT-HOXB4H + G-CSF (Figure 6B) and PBS (Figure 6D) injected monkeys.

Example 8: Effect of Recombinant TAT-HOXB4H Protein on Hematopoietic Recovery in NOD-SCID Mice

[0102] 10^4 human $Lin^-/CD34^+$ cells were injected into irradiated (2.5 Gy) NOD-LtSz-scid/scid (NOD-SCID) mice, along with 10^5 $CD34^-$ irradiated accessory cells. The mice are divided into two groups randomly: one group (n=28) was injected intravenously with 10 μg per kg BW of recombinant TAT-HOXB4H protein twice per day, and the other (n=28) received PBS twice per day. The presence of human $CD45^+$ cells in the peripheral blood cells of all mice was measured periodically by flow cytometry after transplantation. Hematopoietic recovery was evaluated as the number of mice whose human $CD45^+$ cells reached levels of >0.1% in peripheral blood (PB) after transplantation. As shown in Figure 7, improved hematopoietic recovery was observed in the mice injected with recombinant TAT-HOXB4H protein.

Example 9: Effect of Recombinant TAT-HOXB4H Protein on Hematopoietic Recovery in Balb/c mice After Cisplatin Chemotherapy

[0103] 5 week-old Balb/c mice were repeatedly injected intravenously with cisplatin until the number of Ly5 (murine CD45) cells in peripheral blood of the mice decreased to approximately 10% of the original number. The mice treated with cisplatin are divided into two groups randomly: one group (n=28) was injected intravenously with 10 μg per kg BW of recombinant TAT-HOXB4H protein twice per day, and the other (n=28) received PBS twice per day. The presence of Ly5 cells in the peripheral blood cells of all mice was measured periodically by flow cytometry after transplantation. Hematopoietic recovery rate was evaluated as the percentage of the number of Ly5 cells in peripheral blood to

the original number. As shown in Figure 8, improved hematopoietic recovery was observed in the mice injected with recombinant TAT-HOXB4H protein.

[0104] The animal models used in these experiments have been recognized in the art as predictive of results that will be obtained in human patients. *See, e.g.*, Broxmeyer et al. (2005) *The Journal of Experimental Medicine*, 201, 1307-1318; Larochelle et al. (2006) *Blood* 107, 3772-3778.

[0105] Although the invention has been explained in relation to its various embodiments, it is to be understood that many other possible modifications and variations can be made without departing from the spirit and scope of the invention as hereinafter claimed.

Sequence Listing

<110> WU, KOU-JUEY
HUANG, CHI-HUNG

<120> METHOD OF PRODUCING RECOMBINANT TAT-HOXB4H PROTEIN FOR USE AS A STIMULANT OF HEMATOPOIESIS IN VIVO

<130> 10712.0001-00000

<140> 12/042,097
<141> 2008-03-04

<160> 5

<170> PatentIn Ver. 3.3

<210> 1
<211> 57
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic oligonucleotide

<400> 1
tatgcaccac caccaccacc actacggccg caagaaacgc cgccagcgcc gccggcg 57

<210> 2
<211> 53
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic oligonucleotide

<400> 2
ctagcggcgc tggcggcgtt tcttgcggcc gtagtggtgg tggtggtggt gca 53

<210> 3
<211> 840
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic polynucleotide

<400> 3
atgcaccacc accaccacca ctacggccgc aagaaacgcc gccagcgccg ccgcgctagc 60
atggctatga gttctttttt gatcaactca aactatgtcg accccaagtt ccctccatgc 120
gaggaatatt cacagagcga ttacctaccc agcgaccact cgcccgggta ctacgccggc 180
ggccagaggc gagagagcag cttccagccg gaggcgggct tcgggcggcg cgcggcgtgc 240
accgtgcagc gctacgcggc ctgccgggac cctgggcccc cgccgcctcc gccaccaccc 300
ccgccgcccc cgccaccgcc cggtctgtcc cctcgggctc ctgcgccgcc acccgccggg 360
gccctcctcc cggagcccgg ccagcgctgc gaggcggtca gcagcagccc cccgccgcct 420
ccctgcgccc agaacccccc gcaccccagc ccgtcccact ccgcgtgcaa agagcccgtc 480
gtctaccccc ggatgcgcaa agttcacgtg agcacggtaa accccaatta cgccggcggg 540

gagcccaagc gctctcggac cgcctacacg cgccagcagg tcttggagct ggagaaggaa 600
tttcactaca accgctacct gacacggcgc cggagggtgg agatcgccca cgcgctctgc 660
ctctccgagc gccagatcaa gatctggttc cagaaccggc gcatgaagtg gaaaaaagac 720
cacaagttgc ccaacaccaa gatccgctcg ggtggtgcgg caggctcagc cggagggccc 780
cctggccggc ccaatggagg cccccgcgcg ctcctcgagc accaccacca ccaccactga 840

<210> 4
<211> 279
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic polypeptide

<400> 4
Met His His His His His His Tyr Gly Arg Lys Lys Arg Arg Gln Arg
1 5 10 15

Arg Arg Ala Ser Met Ala Met Ser Ser Phe Leu Ile Asn Ser Asn Tyr
20 25 30

Val Asp Pro Lys Phe Pro Pro Cys Glu Glu Tyr Ser Gln Ser Asp Tyr
35 40 45

Leu Pro Ser Asp His Ser Pro Gly Tyr Tyr Ala Gly Gly Gln Arg Arg
50 55 60

Glu Ser Ser Phe Gln Pro Glu Ala Gly Phe Gly Arg Arg Ala Ala Cys
65 70 75 80

Thr Val Gln Arg Tyr Ala Ala Cys Arg Asp Pro Gly Pro Pro Pro Pro
85 90 95

Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Pro Gly Leu Ser Pro Arg
100 105 110

Ala Pro Ala Pro Pro Pro Ala Gly Ala Leu Leu Pro Glu Pro Gly Gln
115 120 125

Arg Cys Glu Ala Val Ser Ser Ser Pro Pro Pro Pro Pro Cys Ala Gln
130 135 140

Asn Pro Leu His Pro Ser Pro Ser His Ser Ala Cys Lys Glu Pro Val
145 150 155 160

Val Tyr Pro Trp Met Arg Lys Val His Val Ser Thr Val Asn Pro Asn
165 170 175

Tyr Ala Gly Gly Glu Pro Lys Arg Ser Arg Thr Ala Tyr Thr Arg Gln
180 185 190

Gln Val Leu Glu Leu Glu Lys Glu Phe His Tyr Asn Arg Tyr Leu Thr
195 200 205

Arg Arg Arg Arg Val Glu Ile Ala His Ala Leu Cys Leu Ser Glu Arg
210 215 220

Gln Ile Lys Ile Trp Phe Gln Asn Arg Arg Met Lys Trp Lys Lys Asp
225 230 235 240

His Lys Leu Pro Asn Thr Lys Ile Arg Ser Gly Gly Ala Ala Gly Ser

245 250 255

Ala Gly Gly Pro Pro Gly Arg Pro Asn Gly Gly Pro Arg Ala Leu Leu
260 265 270

Glu His His His His His His
275

<210> 5
<211> 6
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic 6x his tag

<400> 5
His His His His His His
1 5

Claims

1. A method of producing a TAT-HOXB4H protein comprising:

(a) providing a host cell comprising a vector encoding the protein;

(b) expressing the protein in the host cell;

(c) collecting an impure solution of the expressed protein;

(d) purifying the protein from the solution by:

(i) applying the solution to a HisTrap column;

(ii) washing the HisTrap column;

(iii) eluting the partially purified protein from the HisTrap column to form a partially purified protein solution;

(iv) applying the partially purified protein solution to a MonoSP column;

(v) washing the MonoSP column;

(vi) eluting the purified protein from the MonoSP column in denatured form;

(e) refolding the eluted denatured protein using hydrophobic compounds by:

(i) combining the eluted denatured protein and a solution of hydrophobic compounds to form a solution of protein and hydrophobic compounds;

(ii) desalting the solution of protein and hydrophobic compounds to obtain a desalted protein and hydrophobic compound solution;

(iii) removing the hydrophobic compounds from the desalted protein solution using ultrafiltration.

2. The method of claim 1, wherein the HisTrap column washing buffer comprises 8 M Urea, 20 mM HEPES, 0.5 mM DTT, 100 mM NaCl, pH 8.0 and 10 mM imidazole.

3. The method of claim 1, wherein the eluting buffer to elute the partially purified protein from HisTrap column comprises at least 50 mM imidazole in 8 M Urea, 20 mM HEPES, 0.5 mM DTT, 100 mM NaCl, pH 8.0.

4. The method of claim 1, wherein the eluting buffer to elute the partially purified protein from HisTrap column comprises 50-100 mM imidazole.

5. The method of claim 1, wherein the eluting buffer to elute the partially purified protein from HisTrap column comprises 100-250 mM imidazole.

6. The method of claim 1, wherein the MonoSP column washing buffer comprises 4 M urea, 20 mM HEPES and 50 mM NaCl, pH 6.5.

7. The method of claim 1, wherein the eluting buffer to elute the purified protein from MonoSP column comprises 1. 5 M NaCl and 20 mM HEPES, pH 8.0.

8. The method of claim 1, wherein the hydrophobic compounds comprise Triton X-100, tween-20 or polybenzene compounds.

9. The method of claim 1, wherein the ultrafiltration process is carried out by centricon tube or a stir-cell.

10. The method of claim 1, wherein the purified protein was stored in IMDM or DMEM.

11. The method of claim 9, wherein the ultrafiltration process is carried out using 10K centricon ten times with solution containing different concentrations of beta-cyclodextrin.

12. Use of a TAT-HOXB4H protein produced by the method of claim 1 for the manufacture of a pharmaceutical composition for enhancing the mobilization of HSCs from bone marrow to peripheral blood in a subject.

13. The use of claim 12, wherein the subject in need thereof is a HSC donor.

14. The use of claim 12, wherein the subject in need thereof is a patient having undergone autologous HSC transplantation.

15. The use of claim 12, wherein the subject in need thereof is a G-CSF-insensitive patient.

16. The use of claim 15, wherein the subject suffers from diseases caused by inherited HSC deficiency.

17. The use of claim 16, wherein the subject suffers from inherited aplastic anemia.

18. The use of claim 12, wherein the subject suffers from any one of hematological disorders, solid tumors, and immunological disorders.

19. The use of claim 12, wherein the hematological disorder is selected from the group consisting of lymphomas, leukemias, Hodgkin's diseases and myeloproliferative disorders.

20. Use of a TAT-HOXB4H protein produced by the method of claim 1 for the manufacture of a pharmaceutical composition for improving the recovery time of a subject having undergone HSC transplantation, irradiation or chemotherapy.

21. The use of claim 20, wherein the subject in need thereof is a HSC donor.

22. The use of claim 20, wherein the subject in need thereof is a patient having undergone autologous HSC transplantation.

23. The use of claim 20, wherein the subject in need thereof is a G-CSF-insensitive patient.

24. The use of claim 23, wherein the subject suffers from diseases caused by inherited HSC deficiency.

25. The use of claim 24, wherein the subject suffers from inherited aplastic anemia.

26. The use of claim 20, wherein the subject suffers from any one of hematological disorders, solid tumors, and immunological disorders.

27. The use of claim 26, wherein the hematological disorder is selected from the group consisting of lymphomas, leukemias, Hodgkin's diseases and myeloproliferative disorders.

28. The method of claim 1, further comprising removing a His-tag at the N-terminus of the TAT-HOXB4H.

29. The method of claim 1, further comprising removing a His-tag at the C-terminus of the TAT-HOXB4H.

30. The method of claim 1, further comprising removing His-tags at the both N- and C-termini of the TAT-HOXB4H.

31. The use of claim 12, wherein TAT-HOXB4H does not comprise a His-tag at the N-terminus.

32. The use of claim 12, wherein TAT-HOXB4H does not comprise a His-tag at the C-terminus.

33. The use of claim 12, wherein TAT-HOXB4H does not comprise His-tags at either the N- or C-terminus.

34. The use of claim 20, wherein TAT-HOXB4H does not comprise a His-tag at the N-terminus.

35. The use of claim 20, wherein TAT-HOXB4H does not comprise a His-tag at the C-terminus.

36. The use of claim 20, wherein TAT-HOXB4H does not comprise His-tags at either the N- or C-terminus.