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(54) Title: METHOD OF ISOLATION OF STEM CELL POPULATIONS FROM PERIPHERAL BLOOD USING SIZED-BASED SEPARATION (ELUTRIATION)

(57) Abstract: Disclosed is a method for isolating multiple populations of stem cells in at least one step from a peripheral blood sample without using positive selection, therefore enabling a "no-touch" method to isolate different stem cell populations without contaminating the stem cell populations with positive selection agents (e.g., antibodies, etc.) In particular, the present disclosure relates to flow-rate separation of stem cell populations using elutriation (sized-based separation) to negatively exclude cells based on size. Different fractions or subfractions comprise an increase in yield of Very Small Embryonic-like Stem cells (VSELs), MSCs or HSCs as compared to non-fractionated apheresis product.



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METHOD OF ISOLATION OF STEM CELL POPULATIONS FROM PERIPHERAL
BLOOD USING SIZED-BASED SEPARATION (ELUTRIATION)

FIELD OF THE INVENTION

[001] The subject of the present invention generally relates to cellular separations employing devices having specificity for cell size and density. The biological sample for the cellular source may be blood, such as mobilized peripheral blood and the like. The present invention relates to a system and method for separating particles, and, in particular, provide advantageous methods for separating peripheral blood into desired subsets of stem cell populations.

BACKGROUND OF THE INVENTION

[002] The adult stem cell is an undifferentiated cell that is found in a differentiated tissue. It has the ability to renew itself and become specialized to yield all the cell types of the tissue from which it originated and, in the appropriate environment, can also become a specialized cell of a different tissue. Adult stem cells are capable of self-renewal for the lifetime of the organism.

[003] Sources of adult stem cells have been found in bone marrow, the blood stream, cornea and retina of the eye, the dental pulp of the tooth, liver, skin, gastrointestinal tract, adipose tissue and pancreas. Stem cells offer the possibility of a renewable source of replacement cells and tissues to treat a myriad of diseases, conditions and disabilities. Adult stem cells are relatively quiescent cells, particularly in organisms where cell turnover is low, yet they can mount a rapid and strong response to tissue stress and injury.

[004] As cells designed to withstand crisis and orchestrate repair, stem cells must be especially resilient. Until recently, it had been thought that a stem cell collected from the bone marrow or peripheral blood (a hematopoietic stem cell) could not give rise to cells of a different tissue type, such as nerve cells. However, a number of clinical studies over recent years have affirmed the phenomenon known as plasticity. Plasticity is the ability of adult stem cells to differentiate to other cell types from a specific cell line. In other words, stem cells, from the bone marrow or from the circulating blood do have the ability to differentiate into other cell types such as heart cells or nerve cells.

[005] Because of this plasticity, a human's own adult stem cells are the perfect ethical and moral alternative to stem cells derived from other donors or from embryonic stem cells. Adult stem cell transplants (bone marrow transplants) have been used for over 40 years in successfully

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treating cancers such as leukemia, multiple myeloma and lymphomas, and such transplants have now opened the doors of regenerative and reparative therapeutics.

- [006] Many people diagnosed with a disease which requires a bone marrow transplant are unable to find a healthy match and there is a very low chance of finding a suitable donor. Therefore, it would be advantageous to harvest stem cells from these subjects prior to the need for the bone marrow transplantation arising, where the stem cells can be stored for autologous transplantation at a future timepoint. Accordingly, it is useful to harvest stem cells from healthy subjects when the stem cells are unlikely to have been damaged or programmed for disease. Additionally, stem cells could be harvested from any healthy adult human for future autologous transplantation for a disease or as a regenerative therapy, as well as from adults with a positive family history of cardiac disease, diabetes, cancer, neurologic diseases or autoimmune disorders for future autologous therapeutic use.
- [007] There are tremendous clinical and economic advantages to autologous stem cell transplantation (receiving your own stem cells), including no issues with immune rejection, e.g., including developing host versus graft disease (HVGD) (recipient's immune system sees donor cells as "non-self" and destroys the transplanted cells), or graft versus host disease (GVHD) (donor's T cells reject recipient's cells as "non-self" and destroy cells in the host (recipient)). Additionally, engraftment of subjects with their own stem cells (autologous) is much faster, safer and much less costly than receiving someone else's stem cells (allogenic).
- [008] The ability to identify, characterize and purify subpopulations of stem cells is fundamental to their use in autologous transplantation therapies, as well as in numerous biological and medical applications, often forming the starting point for research protocols and the basis for current and emerging clinical protocols. Cell separation has numerous applications in areas such as medicine, biotechnology, biomedical research, environmental monitoring and bio/chemical warfare defense. For example, cell separation can make possible life-saving procedures such as autologous bone marrow transplantation for the remediation of advanced cancers where the removal of cancer-causing metastatic cells from a patient's marrow is necessitated (Fischer, 1993). In other applications, such as the study of signaling between blood cells (Stout, 1993; Cantrell et al., 1992), highly purified cell subpopulations permit studies that would otherwise be impossible.
- [009] Typically, current approaches to cell sorting most frequently exploit differences in specific immunologic targets (Smeland et al., 1992), or receptor-ligand interactions (Chess et al., 1976), or, in some instances, differences in cell density (Boyum, 1974), to isolate particular

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cells or populations of cells. However, the existing techniques are often inadequate and sorting devices capable of identifying and selectively manipulating stem cells through novel physical properties are therefore desirable. Additionally, techniques for isolating stem cell populations from humans typically isolate only one stem cell population while discarding other stem cell populations, and typically also use positive selection techniques which result in contaminating the collected stem cell population with agents used in the positive selection process.

[0010] The application of the principles of AC electrokinetics has been used for the dielectric characterization of mammalian cells through the method of electrorotation (ROT) (Arnold and Zimmermann, 1982; Fuhr, 1985; Hölzel and Lamprecht, 1992; Wang et al., 1994) and for cell discrimination and sorting (Hagedorn et al., 1992; Huang et al., 1993; Gascoyne et al., 1992; Gascoyne et al., 1994; Huang et al., 1992). In these techniques, cells become electrically polarized when they are subjected to an AC electric field. In ROT, a rotational electrical field is applied and the interaction between the cells' polarization and the applied field results in cell rotation. If that field is inhomogeneous, then the cells experience a lateral dielectrophoretic (DEP) force, the frequency response of which is a function of their intrinsic electrical properties (Gascoyne et al., 1992). In turn, these properties depend strongly on cell composition and organization, features that reflect cell morphology and phenotype. Cells differing in their electrical polarizabilities can thus experience differential forces in the inhomogeneous electric field (Becker et al., 1994; Becker et al., 1995). Analysis of the dielectrophoretic motion of mammalian cells as a function of applied frequency permits cell membrane biophysical parameters, such as capacitance and surface conductance, to be probed. Because DEP force effectively maps biophysical properties into a translational force whose direction and magnitude reflects cellular properties, DEP force may induce separation between particles of different characteristics. For example, DEP has been used on a microscopic scale to separate bacteria from erythrocytes (Markx et al., 1994), viable from nonviable yeast cells (Wang et al., 1993), and erythroleukemia cells from erythrocytes (Huang et al., 1992). However, the differences in the electrical polarizabilities of the cell types in those various mixtures were greater than those to be expected in many typical cell sorting applications.

[0011] Field flow fractionation (FFF) has also been generally employed for separation of matter, utilizing particle density, size, volume, diffusivity, and surface charge as parameters (Giddings, 1993). The technique can be used to separate many different types of matter, from

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a size of about 1 nm to more than about 100 micrometers, which may include, for example, biological and non-biological matter. Separation according to field flow fractionation occurs by differential retention in a stream of liquid flowing through a thin channel. The FFF technique combines elements of chromatography, electrophoresis, and ultracentrifugation, and it utilizes a flow velocity profile established in the thin channel when the fluid is caused to flow through the chamber. Such velocity profile may be, for example, linear or parabolic. A field is then applied at right angles to the flow and serves to drive the matter into different displacements within the flow velocity profile. The matter being displaced at different positions within the velocity profile will be carried with the fluid flow through the chamber at differing velocities. Fields may be based on sedimentation, crossflow, temperature gradient, centrifugal forces, and the like. The technique suffers, however, from producing insufficiently pure cell populations, being too slow, or being too limited in the spectrum of target cells or other matter.

[0012] In the medical field, populations of stem cells can be enriched from peripheral blood.

Whole blood consists of various liquid components and particle components. The liquid portion of blood is largely made up of plasma, and the particle components include red blood cells (erythrocytes) (RBCs), white blood cells (leukocytes) (WBCs), and platelets (thrombocytes) and stem cells, such as but not limited to, hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) and other small stem cells such as Very Small Embryonic-like (VSEL) stem cells. While these constituents have similar densities, their average density relationship, in order of decreasing density, is as follows: red blood cells, white blood cells, platelets, and plasma. In addition, the particle components are related according to size, in order of decreasing size, as follows: white blood cells, red blood cells, and platelets. Most current purification devices rely on density and size differences or surface chemistry characteristics to separate and/or filter the blood components.

[0013] Thus, there exists a need in the art for an easy, quick and efficient method for enriching populations of stem cells from the peripheral blood which is readily available and used in clinical settings.

SUMMARY OF THE INVENTION

[0014] Peripheral blood and mobilized peripheral blood comprise many different types of cells, including stem cells such as, but not limited to, Very Small Embryonic Like Stem cells (VSELs), mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs).

Conventional methods to isolate stem cell populations from the peripheral blood use positive

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and negative selection methods, e.g., using cell-surface markers in methods such as fluorescence activated cell sorting (FACS) and other immunoselection methods to select or exclude a cell population. Additionally, such methods also typically isolate only one stem cell population at a time from the peripheral blood sample.

[0015] In one embodiment, the present invention relates to a method for isolating multiple populations of stem cells in at least one step from a single peripheral blood or mobilized peripheral blood sample without using positive or negative selection, therefore enabling a “no-touch” method to isolate different stem cell populations without contaminating the stem cell populations with positive selection agents (e.g., antibodies, etc). In particular, in one embodiment, the present invention relates to a method for the flow-rate separation of stem cell populations using elutriation (size-based separation) to negatively exclude cells based on size, to separate the peripheral blood into various elutriation fractions, each comprising a different stem cell of interest. The inventors have demonstrated that different fractions or subfractions comprise an increase in yield of Very Small Embryonic Like Stem cells (VSELs), MSCs or HSCs as compared to non-fractionated apheresis product. In some embodiments, the isolated stem cell populations obtained by the methods as disclosed herein provide a library of different stem cell types from a particular individual which can be maintained in culture and/or cryopreserved for future use, e.g., for use alone, or selectively recombined (e.g., custom mixing) for individualized autologous therapeutic applications in regenerative therapy. Additionally, in some embodiments, the isolated stem cell populations also provide a pool of different stem cell populations for personalized cell-based assays to assess the effect of a persons diet, pharmacogenetics, neurochemicals, and lifestyle on the function and viability of different stem cell populations, either alone or as a combination of different stem cells.

[0016] Accordingly, the present invention generally relates to methods and compositions for the separation or fractionation of peripheral blood into a plurality of fractions which comprise different stem cell populations from a single peripheral blood sample from a subject in a single collection process.

[0017] The present invention generally relates to a quick, easy, effective and inexpensive method for isolating stem cell populations from peripheral blood, such as peripheral blood obtained from a human subject without using positive selection techniques. In some embodiments, the peripheral blood is mobilized peripheral blood, and in some embodiments, the peripheral blood is pre-processed by apheresis to generate an apheresis product.

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[0018] In particular, the present invention relates to methods for isolating a plurality of different stem cell populations from a peripheral blood sample without the need to add positive selection agents to the peripheral blood sample. Thus, the inventors have discovered a method to isolate a plurality of different stem cell populations from a single sample without contaminating the collected stem cell populations with positive selection agents.

[0019] In some embodiments, the present invention provides methods to fractionate peripheral blood into a plurality of fractions, wherein each fraction comprises a distinct target population of stem cells. Thus, the present invention relates to a method for enriching for a target population of stem cells obtained from peripheral blood without using positive selection techniques. In particular, the present invention relates to a method of separating a peripheral blood sample into a plurality of different fractions, enabling the enrichment of a plurality of different target stem cell populations in different fractions. In some embodiments, the resulting fractions comprising a target stem cell population can be further separated into subsets or subpopulations of desired target stem cells, if desired. In some embodiments, the collected fraction comprising the target stem population can be subject to positive selection methods to enrich for the desired stem cell population.

[0020] In cell-based therapy, it is often necessary to both purify and concentrate subpopulations of stem cells in a sample, such as a peripheral blood sample. In some stem cell therapies, there is a desire to collect bone marrow (BM)-derived, and/or peripheral blood (PB)-derived stem cells from a subject for future use in an autologous cell replacement or autologous cell-based regenerative therapies.

[0021] Peripheral blood, including mobilized peripheral blood, comprises a variety of different types of stem cells, including hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) and other stem cell populations, including but not limited to Very Small Embryonic Like (VSEL) stem cells. Typical conventional methods to isolate such stem cell populations, such as HSCs, MSCs and VSELs, from peripheral blood samples involve positive selection methods. Such selection methods include methods based on cell-surface markers, e.g., immunoselection, which involves separation and isolation based on the binding of an affinity molecule such as an antibody or cell-surface ligand to the surface of the target stem cell to be separated, where the antibody is used to isolate the target stem cell. Such positive separation methods are commonly known in the art, and include, without limitation, fluorescence cell sorting (FACS), immunodensity separation, immunomagnetic separation and the like.

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[0022] However, these conventional methods for positive or negative selection of different stem cell populations from a peripheral blood sample result in the contamination of both the peripheral blood sample and the isolated stem cell population with selection agents, such as antibodies. Furthermore, these selection methods are also expensive, time consuming and slow procedures that often require skilled professionals or specific training of technicians and/or specialized equipment. Additionally, conventional selection methods to isolate stem cells populations from peripheral blood samples often only enrich for one target stem cell population from a peripheral blood sample and discard other potentially useful non-selected, or non-enriched stem cell populations which are also present in the peripheral blood sample.

[0023] Accordingly, the present invention directed to a method to isolate or enrich for numerous stem cell populations from a peripheral blood sample without using positive selection techniques as disclosed herein, has numerous advantages over existing methods for stem cell enrichment from peripheral blood, including, but not limited to, (i) enriching for multiple different stem populations from a single peripheral blood sample, enabling the collection of a variety of different stem cell populations present in peripheral blood and avoiding having to discard potentially valuable or useful stem cell populations, (ii) enriching for stem cell populations in the peripheral blood without the need to add additional agents, therefore avoiding contamination of collected stem cell population or the peripheral blood sample with agents, (e.g., agents which may not meet with FDA approval should the enriched stem cell population be subsequently used in autologous or allogenic transplantation in humans), (iii) providing a method to enrich for multiple different stem cell populations from peripheral blood which can be readily carried out by personnel of ordinary skill with little or no training, which uses a quick, easy and inexpensive method, and can be readily carried out in multiple locations without the need for specialized equipment, reagents or highly trained personnel.

[0024] In some embodiments, the present invention provides methods for the isolation of a plurality of different stem cell populations without the contamination of agents typically used for positive selection or isolation of stem cell populations, e.g., without contamination with antibodies or other agents used in immunoselection methods, or without the need to isolate and select the cells based on cell-surface markers, such as done in conventional stem cell isolation methods that use immunoselection, such as fluorescent cell sorting (FACS) or magnetic bead separation after interaction of the target cell with a labeled antibody. Immunoselection methods include affinity methods with antibodies labeled to magnetic

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beads, biodegradable beads, non-biodegradable beads and antibodies panned to surfaces including dishes and combinations of such methods.

[0025] Accordingly, the present invention generally relates to peripheral blood fractionation comprising the separation of cells present in a peripheral blood (PB) sample into distinct fractions of cells, where each fraction is heterogenous population of cells, where some fractions comprise at least one distinct target stem cell population. In some embodiments, each PB fraction comprising a heterogenous population of cells can be further fractionated, for example into distinct homogenous target stem cell populations. For example, a PB fraction comprising a heterogenous population of cells which comprises at least two populations of target stem cell populations (e.g. HSCs and MSCs) can be further fractionated onto a substantially pure population of HSCs, a substantially pure population of MSCs and a heterogeneous population of cells which are neither HSCs or MSCs. In another example, a PB fraction comprising a heterogenous population of cells which comprises a population of target stem cells (e.g., VSEL stem cells) can be further fractionated onto a substantially pure population of VSELs and a heterogeneous population of non-VSEL cells.

[0026] Accordingly, the present invention relates to methods for the isolation of a plurality of target stem cell populations from a single peripheral blood sample without using positive selection techniques, and thus avoiding contaminating the isolated target stem cell population or the peripheral blood sample with an agent which would otherwise be necessary for the selection or enrichment of a target stem cell population.

[0027] In some embodiments, the peripheral blood fractionation is achieved by elutriation, wherein the flow rate through the elutriation apparatus determines which cells from the peripheral blood are collected in a specific PB fraction. In some embodiments, an apheresis product from peripheral blood is processed at different flow rates through an ELUTRA® apparatus, or other suitable elutriation apparatus or machine. In some embodiments, the flow rates used in the elutriation procedure is maintained and centripetal acceleration force (*i.e.*, “g”) is varied to fractionate the peripheral or apheresed blood.

[0028] The inventors demonstrate using the method as disclosed herein the ability to enrich for a population of VSEL stem cells from peripheral blood, where a non-fractionated apheresis peripheral blood sample which comprises about 0.01% VSELs is enriched for VSELS at a flow rate of 50ml/minute to generate a fractionated PB sample which comprises about at least 0.3% VSEL stem cells, demonstrating at least a 30-fold enrichment in a population of VSELS from the peripheral blood. Accordingly, in some embodiments, a PB fraction collected from a

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flow rate of 50ml/minute comprises a target stem cell population comprising VSEL stem cells, as disclosed in the Examples herein. In some embodiments, the percentage of VSELs in a PB fraction obtained from a flow rate of 50ml/minute is greater than about 0.1%, or about 0.2%, or about 0.3% or about 0.4% or about 0.5%, or greater than 0.5%. In some embodiments, the PB fraction collected from a flow rate of 50ml/minute is enriched in VSELs by about 15-fold, or about 20-fold, or about 30-fold, or about 40-fold, or about 50-fold or greater than about 50-fold as compared to non-fractionated peripheral blood.

[0029] In some embodiments, a PB fraction collected from a flow rate of greater or equal to about 90ml/min comprises a target stem cell population comprising HSCs and MSCs, as disclosed in the Examples herein. In some embodiments, the percentage of HSCs or MSCs in a PB fraction obtained from a flow rate of greater or equal to about 90ml/min is greater than about 15%, or about 20%, or about 30% or about 40% or about 50% or greater than about 50%, or any integer between 15% and 50%, or above about 50%.

[0030] In one aspect, the present invention provides a method for separating peripheral blood to obtain at least four target cell populations without using positive selection techniques, comprising: (a) flowing a peripheral blood sample from a subject through a fluid chamber of an elutriation apparatus, wherein a first flow rate is selected wherein cells in the peripheral blood larger than the smallest cells will flow through, and smaller cells are retained in the fluid chamber; (b) collecting the retained small cells from step (a) in the fluid chamber without subjecting the retained cells to a positive selection step, to obtain a first target cell population; (c) recirculating the peripheral blood sample comprising the non-retained cells through the fluid chamber of the elutriation apparatus, and increasing the flow rate of the remaining peripheral blood sample, wherein cells in the peripheral blood larger than the smallest cells will flow through, and smaller cells are retained in the fluid chamber; (d) collecting the retained small cells in the fluid chamber from step (c) without subjecting the retained cells to a positive selection step, to obtain a second target cell population; and (e) repeating steps (c) to (d) until all the cells from the peripheral blood sample are collected into desired cell populations.

[0031] In some embodiments, the first flow rate is 20ml/minute or less and the first target cell population comprises platelets. In some embodiments, an increased flow rate is 50ml/minute or less and the target cell population comprises a population of Very Small Embryonic-like (VSEL) stem cells. In some embodiments, an increased flow rate is 70ml/minute or less and the target cell population comprises a population of red blood cells (RBC). In some

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embodiments, an increased flow rate is 90ml/minute or less and the target cell population comprises a population of white blood cells (WBC). In some embodiments, an increased flow rate is 105ml/minute or less, and the target cell population comprises a population of hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs).

[0032] In some embodiments, the methods as disclosed herein do not use a positive selection technique such as immunoselection or immunodensity selection, e.g., FACS. In some embodiments, the methods as disclosed herein do not use a positive selection technique which uses antibodies, or antibody fragments. In some embodiments, a retained cell population (e.g., a fraction of PB which comprises a target stem cell population) can be subjected to positive selection techniques to enrich for a desired target population of stem cells in the retained cell population.

[0033] In some embodiments, the method for separating peripheral blood to obtain at least four target cell populations as disclosed herein uses a peripheral blood sample, such as a human peripheral blood sample, including a mobilized peripheral blood sample. In some embodiments, a peripheral blood sample is obtained from a human subject after administration of a mobilizing agent, such as, for example, where a human subject has been administered a mobilizing agent for at least 2 days, or four days or less. In some embodiments, the peripheral blood sample is an apheresis product.

[0034] One aspect of the present invention relates to a method for separating peripheral blood to obtain at least four target cell populations without using positive selection techniques comprising: (a) flowing a peripheral blood sample from a subject through a fluid chamber of an elutriation apparatus, wherein a first flow rate is selected wherein cells in the peripheral blood larger than the smallest cells will flow through, and smaller cells are retained in the fluid chamber; (b) collecting the retained small cells from step (a) in the fluid chamber without subjecting the retained cells to a positive selection step, to obtain a first target cell population; (c) recirculating the peripheral blood sample comprising the non-retained cells through the fluid chamber of the elutriation apparatus, and increasing the flow rate of the remaining peripheral blood sample, wherein cells in the peripheral blood larger than the smallest cells will flow through, and smaller cells are retained in the fluid chamber; (d) collecting the retained small cells in the fluid chamber from step (c) without subjecting the retained cells to a positive selection step, to obtain a second target cell population; and (e) repeating steps (c) to (d) until all the cells from the peripheral blood sample are collected into desired cell populations.

[0035] In some embodiments, the first flow rate is 20ml/minute or less and the first target cell population comprises platelets. In some embodiments, an increased flow rate is 50ml/minute or less and the target cell population comprises a population of Very Small Embryonic-like (VSEL) stem cells. In some embodiments, an increased flow rate is 70ml/minute or less. In some embodiments, an increased flow rate is 90ml/minute or less and the target cell population comprises a population of red blood cells (RBC) and white blood cells (WBC). In some embodiments, an increased flow rate is greater than 90ml/minute or less, and the target cell population comprises a population of hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). In some embodiments, an increased flow rate is about 105ml/minute or less, and the target cell population comprises a population of hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs).

[0036] In some embodiments, the methods as disclosed herein do not use a positive selection technique, such as immunoselection or immunodensity selection, e.g., FACS. In some embodiments, the methods as disclosed herein do not use a positive selection technique that uses antibodies or antibody fragments.

[0037] In some embodiments, a retained cell population can be subjected to negative selection techniques to enrich for a desired target population of stem cells in the retained cell population. In some embodiments, the negative selection techniques use immunoselection and immunodensity selection methods. In some embodiments, the negative selection techniques are used to remove undesired cells and components from the target stem cell population.

[0038] In some embodiments, the method can be used to obtain a plurality of stem cell populations from a peripheral blood sample such as a human peripheral blood sample, including a mobilized peripheral blood sample. In some embodiments, a peripheral blood sample is obtained from a human subject after administration of a mobilizing agent, for example, where a human subject has been administered a mobilizing agent for at least 1 day, but less than four days. In an embodiment of the invention, a mobilizing agent is administered for two days. In another embodiment, the mobilizing agent is administered for three days. In some embodiments, the peripheral blood sample is an apheresis product.

[0039] Another aspect of the present invention relates to a method for treating a disease or disorder in a subject with at least one autologous stem cell population comprising: (a) utilizing at least one stem cell population from a peripheral blood sample, wherein the peripheral blood sample is obtained from a subject who has been administered a mobilizing agent for 4 days or

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less, and wherein the at least one stem cell population was enriched using a flow-rate separation process without using a positive selection technique; and (b) administering at least one stem cell population to the subject to treat the disease or disorder.

[0040] In some embodiments, at least one stem cell population obtained using the methods as disclosed herein has been cryopreserved. In some embodiments, at least one stem cell population comprises non-stem cells, for example, a stem cell population comprising at least 10% of a target stem cell population. In some embodiments, at least one stem cell population is a substantially pure population of a target stem cell population. In some embodiments, at least one stem cell population has been expanded in vitro prior to administering to the subject, such as a human subject. In some embodiments, the methods enable collecting multiple stem cell populations from peripheral blood, wherein at least one stem cell population is Very Small Embryonic-like (VSEL) stem cells, and at least one stem cell population is MSCs, and at least one stem cell population is HSCs. In some embodiments, the at least one stem cell population administered to the subject is a combination of cells comprising any combination selected from the group of Very Small Embryonic-like (VSEL) stem cells, MSCs or HSCs.

[0041] In some embodiments, the present invention provides a method for separating a target cell population from peripheral blood comprising:

(a) flowing a peripheral blood sample from a subject through a fluid chamber of an elutriation apparatus at a first flow rate, wherein the first flow rate is selected so that cells in the peripheral blood sample smaller than the target cell population flow through the fluid chamber and the target cell population is retained in the fluid chamber;

(b) increasing the first flow rate to a second flow rate so that the target cell population flows through the fluid chamber; and

(c) collecting the target cell population as it flows through the fluid chamber; where steps (a), (b), and (c) do not comprise the use of positive selection techniques.

[0042] In some embodiments, (i) the first flow rate is selected so that cells in the peripheral blood sample smaller than Very Small Embryonic Like stem cells (VSELs) flow through the fluid chamber and VSELs are retained in the fluid chamber and (ii) the second flow rate is selected so that VSELs flow through the fluid chamber.

[0043] In some embodiments, (i) the first flow rate is about 35 ml/min, the second flow rate is about 50-70 ml/min, and VSELs are collected.

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[0044] In some embodiments, (i) the first flow rate is selected so that cells in the peripheral blood sample smaller than mesenchymal stem cells (MSCs) flow through the fluid chamber and MSCs are retained in the fluid chamber and (ii) the second flow rate is selected so that MSCs flow through the fluid chamber.

[0045] In some embodiments, (i) the first flow rate is about 100 ml/min, the second flow rate is about 110-120 ml/min, and MSCs are collected.

[0046] In some embodiments, (i) the first flow rate is selected so that cells in the peripheral blood sample smaller than hematopoietic stem cells (HSCs) flow through the fluid chamber and HSCs are retained in the fluid chamber and (ii) the second flow rate is selected so that HSCs flow through the fluid chamber.

[0047] In some embodiments, (i) the first flow rate is about 90 ml/min, the second flow rate is about 100 ml/min, and HSCs are collected.

[0048] In some embodiments, the positive selection technique is immunoselection or immunodensity selection.

[0049] In some embodiments, the peripheral blood sample is a human peripheral blood sample.

[0050] In some embodiments, the peripheral blood sample is a mobilized peripheral blood sample.

[0051] In some embodiments, the peripheral blood sample is obtained from a subject who has been administered a mobilizing agent for 4 days or less.

[0052] In some embodiments, the collected target cell population is cryopreserved.

[0053] In some embodiments, at least one of the collected target cell populations is administered to a subject.

[0054] In some embodiments, at least one of the collected target cell populations are administered to a subject from whom the peripheral blood was originally obtained.

[0055] In some embodiments, at least one of the collected target cell populations has been expanded *in vitro* prior to administering to the subject.

[0056] In some embodiments, the subject is a human subject.

[0057] In some embodiments, at least one of the collected target cell populations is VSELs.

[0058] In some embodiments, at least one of the collected target cell populations is MSCs.

[0059] In some embodiments, at least one of the collected target cell populations is HSCs.

[0060] In some embodiments, the present invention provides a method for separating target cell populations from peripheral blood comprising:

(a) flowing a peripheral blood sample from a subject through a fluid chamber of an elutriation apparatus at a first flow rate, wherein the first flow rate is selected so that a first

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target cell population flows through the fluid chamber and is collected while a first remainder of the peripheral blood sample is retained in the fluid chamber; and

(b) increasing the first flow rate to a second flow rate so that a second target cell population flows through the fluid chamber and is collected while a second remainder of the peripheral blood sample is retained in the fluid chamber;

where steps (a) and (b) do not comprise the use of positive selection techniques.

[0061] In some embodiments, the method further comprises:

(c) increasing the second flow rate to a third flow rate so that a third target cell population flows through the fluid chamber and is collected while a third remainder of the peripheral blood sample is retained in the fluid chamber;

where step (c) does not comprise the use of positive selection techniques.

[0062] In some embodiments, the method further comprises:

(d) increasing the third flow rate to a fourth flow rate so that a fourth target cell population flows through the fluid chamber and is collected while a fourth remainder of the peripheral blood sample is retained in the fluid chamber;

where step (d) does not comprise the use of positive selection techniques.

[0063] In some embodiments, the method further comprises:

(e) increasing the fourth flow rate to a fifth flow rate so that a fifth target cell population flows through the fluid chamber and is collected while a fifth remainder of the peripheral blood sample is retained in the fluid chamber;

where step (e) does not comprise the use of positive selection techniques.

[0064] In some embodiments, the method further comprises:

(f) increasing the fifth flow rate to a sixth flow rate so that a sixth target cell population flows through the fluid chamber and is collected while a sixth remainder of the peripheral blood sample is retained in the fluid chamber;

where step (f) does not comprise the use of positive selection techniques.

[0065] In some embodiments, the method further comprises:

(g) increasing the sixth flow rate to a seventh flow rate so that a seventh target cell population flows through the fluid chamber and is collected while a seventh remainder of the peripheral blood sample is retained in the fluid chamber;

where step (g) does not comprise the use of positive selection techniques.

[0066] In some embodiments, the first flow rate is selected so that the first target cell population is cells smaller than Very Small Embryonic Like stem cells (VSELs) and (ii) the second flow rate is selected so that the second target cell population is VSELs.

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- [0067] In some embodiments, (i) the first flow rate is about 35 ml/min, the second flow rate is about 50-70 ml/min, and VSELs are collected.
- [0068] In some embodiments, (i) the third flow rate is selected so that the first target cell population is cells smaller than hematopoietic stem cells (HSCs) and (ii) the fourth flow rate is selected so that the second target cell population is HSCs.
- [0069] In some embodiments, (i) the third flow rate is about 90 ml/min, the fourth flow rate is about 100 ml/min, and HSCs are collected.
- [0070] In some embodiments, (i) the fourth flow rate is selected so that the first target cell population is cells smaller than mesenchymal stem cells (MSCs) and (ii) the fifth flow rate is selected so that the second target cell population is MSCs.
- [0071] In some embodiments, (i) the third flow rate is about 100 ml/min, the fourth flow rate is about 110-120 ml/min, and MSCs are collected.
- [0072] In some embodiments, (i) the first flow rate is about 35 ml/min and the first target cell population is platelets;
(ii) the second flow rate is about 50 ml/min and the second target cell population is VSELs;
(iii) the third flow rate is about 70 ml/min and the third target cell population is VSELs;
(iv) the fourth flow rate is about 90 ml/min and the fourth target cell population is red blood cells;
(v) the fifth flow rate is about 100 ml/min and the fifth target cell population is HSCs;
(vi) the sixth flow rate is about 110 ml/min and the sixth target cell population is MSCs; and
(vii) the seventh flow rate is about 120 ml/min and the seventh target cell population is MSCs.
- [0073] In some embodiments, the positive selection technique is immunoselection or immunodensity selection.
- [0074] In some embodiments, the peripheral blood sample is a human peripheral blood sample.
- [0075] In some embodiments, the peripheral blood sample is a mobilized peripheral blood sample.
- [0076] In some embodiments, the peripheral blood sample is obtained from a subject who has been administered a mobilizing agent for 4 days or less.
- [0077] In some embodiments, the collected target cell population is cryopreserved.
- [0078] In some embodiments, at least one of the collected target cell populations is administered to a subject.
- [0079] In some embodiments, at least one of the collected target cell populations are administered to a subject from whom the peripheral blood was originally obtained.

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[0080] In some embodiments, at least one of the collected target cell populations has been expanded *in vitro* prior to administering to the subject.

[0081] In some embodiments, the subject is a human subject.

[0082] In some embodiments, at least one of the collected target cell populations is VSELs.

[0083] In some embodiments, at least one of the collected target cell populations is MSCs.

[0084] In some embodiments, at least one of the collected target cell populations is HSCs.

[0085] In some embodiments, the present invention provides a method for separating target cell populations from peripheral blood comprising:

(a) flowing a peripheral blood sample from a subject through a fluid chamber of an elutriation apparatus at a first flow rate, wherein the first flow rate is selected so that a first target cell population flows through the fluid chamber and is collected while a first remainder of the peripheral blood sample is retained in the fluid chamber;

(b) increasing the first flow rate to a second flow rate so that a second target cell population flows through the fluid chamber and is collected while a second remainder of the peripheral blood sample is retained in the fluid chamber; and

(c) optionally, repeating step (b) until a desired number of target cell populations are collected;

where steps (a), (b), and (c) do not comprise the use of positive selection techniques.

[0086] In some embodiments, the present invention provides VSELs, MSCs, or HSCs product by one of the methods disclosed above.

BRIEF DESCRIPTION OF THE DRAWINGS

[0087] Figure 1. Figure 1 shows a graph displaying that Very Small Embryonic-like Stem Cells (VSELs) can be enriched by size-based separation (elutriation) of various cell fractions in the apheresis product. Fraction 1 (Flow rate 20 mL/min), Fraction 2 (50 mL/min), Fraction 3 (70 mL/min), Fraction 4 (90 mL/min), Fraction 5 (>90 mL/min). Fraction 2 is highly enriched in VSELs and can be used to harvest purified populations of VSELs for clinical applications.

[0088] Figure 2. Figure 2 is a photograph showing the size-based separation of an apheresis product from peripheral blood into 5 separate fractions.

[0089] Figure 3A-F shows the enrichment of MSCs from apheresed human peripheral blood. G-CSF mobilized apheresed blood was separated using an ELUTRA® Cell Separation System. MSCs were enriched in fractions 4 and 5. Figure 3A – apheresed blood. Figure 3B – Fraction 1. Figure 3C – Fraction 2. Figure 3D – Fraction 3. Figure 3E – Fractions 4 and 5. Figure 3F – Fraction 6.

[0090] Figure 4A-E shows cells from elutriation fractions that were plated in a CFU-F assay.

Colonies of adherent cells developed from fractions 4 and 5. Figure 4A – Fraction 2. Figure 4B – Fraction 3. Figure 4C – Fractions 4 and 5. Figure 4D – Higher magnification of Fractions 4 and 5. Figure 4E – Higher magnification of Fractions 4 and 5.

[0091] Figure 5A-B shows the mobilization of MSCs into peripheral blood. Patients received two consecutive shots of G-CSF subcutaneously. The degree of mobilization of mesenchymal stem cells was monitored at day 0 prior to receiving a G-CSF injection, day 3 after receiving the second G-CSF injections, and in the apheresis product. Figure 5A: MSC mobilization assessed by evaluation of CD45⁺/CD31⁻/CD105⁺/CD73⁺ cells. Figure 5B: MSC mobilization assessed by detection of CD45⁺/CD34⁺/CD90⁺/CD105⁺/CD29⁺ cells. A group of “n = 28” donor samples were analyzed.

[0092] Figure 6A-B shows FACS analysis of MSC phenotype in ELUTRA® fractions. Figure 6A: Fractions 4 and 5 were enriched for CD45⁺/CD105⁺ cells compared to other fractions. Figure 6B: Fractions 4 and 5 showed an increase in the proportion of CD45⁺/CD105⁺, CD45⁺/CD90⁺, and CD271⁺ cells as compared to the apheresis product starting population.

DETAILED DESCRIPTION OF THE INVENTION

[0093] In one embodiment, the present invention enables flexibility to optimize the recovery of specific target stem cell populations from a peripheral blood sample, where a plurality of different target stem cell populations are enriched with yields that have a high number of cells with low cross-cellular contamination of non-stem cells or non-target stem cells.

[0094] One aspect of the present invention relates to the use of elutriation to rapidly and reliably separate bulk stem cell populations from peripheral blood, where each target stem cell population has a different sedimentation coefficient (size and density) as to other distinct populations of target stem cells, allowing enrichment of different target stem cell populations with virtually no loss in either recovery or viability of the target stem cell populations.

[0095] In some embodiments, target stem cells obtained from peripheral blood according to the methods as disclosed herein are useful for cell therapy. In some stem cell therapies, there is a need to use bone marrow (BM)- or peripheral blood (PB)-derived stem cells for cell replacement or regenerative therapies. Methods for separating stem cells and non-stem cells can be divided into three basic types:

[0096] (a) separation based on a cell's physical properties such as cell density and size, which includes methods based on differential migration in a fluid flow (field flow fractionation) or

in a sedimentation field (centrifugation) or in a fluid flow in the presence of a sedimentation field (elutriation). Differential sedimentation can be performed in combination with a solution or hydrogels of varying inherent densities, either in discontinuous or continuous (gradient) form.

[0097](b) Separation based on cell-surface properties, such as unit charge (electrophoresis) or interfacial free energy (phase portion) or presence of specific surface molecular groups (which interact with various affinity surfaces). Examples of separation based on specific surface molecular groups include use of affinity monoclonal or polyclonal antibodies to cell surface receptors or other determinants. Affinity methods involve negative selection (e.g., interaction with the cell surface receptor and the affinity monoclonal antibody to remove unwanted populations of cells) or positive selection (interaction with the cell surface receptors and the affinity molecule, e.g., monoclonal antibody to select for the desired target cell).

[0098](c) selection based upon a combination of a cell's physical properties and surface properties, e.g., selection based on density gradient media used to isolate a population of cells in combination with cell-surface specific antibodies to recognize target cells or an undesired subpopulation of the cells.

[0099] However, isolation of stem cells has typically been based on cell-surface properties.

Moreover, conventional methods to isolate stem cell populations, such as HSCs, MSCs and VSELs, from a peripheral blood sample typically involve cell-surface based separation methods, such as immunoselection, where separation and isolation of a particular stem cell population is based on the binding of an affinity molecule, such as an antibody or cell-surface ligand to the surface of the target stem cell to be separated. Such separation methods are commonly known in the art, and include without limitation, fluorescence cell sorting (FACS), immuodensity separation and immunomagnetic separation and the like. Thus, conventional methods for isolation of different stem cell populations from peripheral blood require contamination of the peripheral blood sample with antibodies, and are expensive as well as slow, and often require skilled professionals or specific training of technicians and/or specialized equipment. Additionally, conventional methods often enrich for only one stem cell population in a peripheral blood sample and discard other potentially useful non-selected, or non-enriched, stem cell populations present in the peripheral blood sample.

[00100] Additionally, problems exist in the conventional methods to enrich for stem cells from peripheral blood, such as contamination of other non-target stem cells, which can interfere

with various cell separation and/or cell selection techniques and later cultivation of the selected target stem cells for therapeutic use. Thus, it is desirable to separate the stem cells from peripheral blood into the desired subsets of stem cell populations and remove or reduce the number of non-target stem cell populations in each subset of stem cells. The invention provides a method for separating desired populations of stem cells that can be performed using large amounts of input cells without the need for a selection process using cell surface markers or reagents associated with such a process. The instant invention provides separation parameters and demonstrates that several types of stem cells can be effectively enriched from other cellular components of peripheral and apheresed blood.

[00101] In some embodiments, the resulting fractions comprising a target stem cell population can be further separated into subsets of desired cells for collection, if desired.

[00102] It has previously been reported that separation of G-CSF-mobilized PBSC transplants by counterflow centrifugal elutriation resulted in a modest enrichment of CD34+ cells but no loss of primitive hematopoietic progenitors (see Kwekkeboom et al., *British Journal of Haematology*, 1997, 99; 47-55). Kwekkeboom et al. discuss the use of counterflow centrifugal elutriation (CCE) for reduction of the number of non-stem cells in autologous G-CSF-mobilized peripheral blood stem cell (PBSC) transplants. By cell size-monitored CCE, small cells could be rapidly separated from the hematopoietic progenitor cells present in leukapheresis product (LP) samples. However, unlike the present invention, Kwekkeboom et al. do not discuss the use of CCE for separation of different stem cell populations from a peripheral blood sample. More particularly, Kwekkeboom found no indication of preferential co-elutriation of primitive stem cells with any fraction, including small cell fractions.

[00103] Micklethwaite et al. discuss a clinical-scale elutriation using the ELUTRA® to enrich for monocytes for immunotherapy protocols, and performed analysis of the four (F1–F4) non-monocyte fractions derived from this process, and discuss that ELUTRA® enriches cell subsets that may be incorporated into and enhance existing immunotherapy and stem cell transplantation protocols. (Micklethwaite et al., *Clinical-scale elutriation as a means of enriching antigen-presenting cells and manipulating alloreactivity*, *Cytotherapy*; 2009, 11; 218-228). Micklethwaite et al. discuss using FACS to analyze enrichment of T, B, natural killer (NK) and dendritic cells (DC) from the different ELUTRA® fractions, such as F3-F4. However, unlike the present invention, Micklethwaite et al. discuss use of ELUTRA® to enrich for hematopoietic cells, such as dendritic cells or natural killer cells, but not enrichment of stem cell populations from mobilized human peripheral blood.

Definitions

[00104] For convenience, certain terms employed herein in the specification, examples and appended claims are collected here. Unless stated otherwise, or implicit from context, the following terms and phrases include the meanings provided below. Unless explicitly stated otherwise, or apparent from context, the terms and phrases below do not exclude the meaning that the term or phrase has acquired in the art to which it pertains. The definitions are provided to aid in describing particular embodiments and are not intended to limit the claimed invention because the scope of the invention is limited only by the claims. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[00105] The term “apheresis” as used herein refers to the process or procedure in which blood is drawn from a donor subject and separated into its components, some of which are retained, such as plasma, platelets and/or stem cell populations, and the remainder returned by transfusion to the donor subject. This process can also be referred to in the art as hemapheresis or pheresis. The forms of apheresis include: Plasmapheresis -- to harvest plasma (the liquid part of the blood); Leukapheresis -- to harvest leukocytes (white blood cells); Granulocytophoresis -- to harvest granulocytes (neutrophils, eosinophils, and basophils); Lymphocytophoresis -- to harvest lymphocytes; Lymphoplasmapheresis - to harvest lymphocytes and plasma; Plateletpheresis (thrombocytophoresis) - to harvest platelets (thrombocytes). Apheresis takes longer than a whole blood donation. A whole blood donation takes about 10-20 minutes to collect the blood, while an apheresis donation may take about 1-2 hours.

[00106] The term “apheresis product” as used herein refers to the heterogeneous population of cells collected from the process of apheresis. As disclosed herein, the cells present in an apheresis product may be separated using elutriation.

[00107] The term “elutriation” as used herein refers to the separation or enrichment of cells, e.g., stem cells from peripheral blood on the basis of their differential sedimentation rate. Elutriation as used herein is a noninvasive method for separating large numbers of cells on the basis of their size and mass. Also, elutriation allows for separating mixed populations of cells, in particular stem cells at different stages of the cell division cycle without perturbing stem cell metabolism or using synchronizing agents

[00108] The terms “enriching” or “enriched” or “enrich” are used interchangeably herein and mean that the yield (i.e., fraction) of cells of one type is increased by at least 10% over the

fraction of cells of that type in the starting culture or preparation.

[00109] The terms “separation” or “selection” as used herein refer to isolating different cell types into one or more populations and collecting the isolated population as a target cell population which is enriched in a specific target stem cell population. Selection can occur using positive selection for the enriched cell population or negative selection to discard non-target cell populations.

[00110] The term “purify” as used herein means to separate away unwanted components.

[00111] The term “positive selection” as used herein refers to a method where the desired stem cells are targeted for selection, e.g., using a monoclonal antibody to a specific cell surface antigen on the desired or target stem cell.

[00112] The term “negative selection” as used herein refers to targeting unwanted or non-target stem cells for depletion, e.g., using monoclonal antibodies to specific cell surface antigens. In negative selection, desired cells or target cells are not labeled with antibody.

[00113] The term “remove” as used herein refers to separate and select and set aside (e.g., for retaining or discarding). Thus, a target population of cells can be removed from a mixed population of cells with the intent of keeping them or discarding them.

[00114] The term “immunoselection” as used herein refers to a process where cells, e.g., stem cells, are labeled using monoclonal antibodies and separated by selecting for the antibody, using a marker on the antibody, such as fluorescence, or magnetic particles.

[00115] The term “immunodensity selection” as used herein refers to a process where unwanted cells, e.g., non-target cells are targeted with monoclonal antibody-based reagents, and pellet when centrifuged over density medium.

[00116] The term “fractionation” as used herein refers to the production of homogeneous sets of stem cell populations from a population of heterogeneous stem cells.

[00117] The term “peripheral blood” as used herein refers to whole blood obtained from a subject.

[00118] The terms “mobilized peripheral blood” as used herein refers to peripheral blood which is obtained from a subject where the subject has been administered a mobilizing agent for enhancing the number of stem cells in the peripheral blood, by increasing the migration of stem cells from the bone marrow (e.g., increasing BM-derived stem cells) into the peripheral blood, or increasing the proliferation of stem cell present in the peripheral blood (e.g., increasing number of PB-derived stem cells). Mobilization may be effectuated in a subject by administering an effective amount of a mobilizing agent, e.g., by a combination of

chemoattractants (e.g., cytokines) and loss of adhesiveness of pools or populations of stem cells residing in stem cell niches in peripheral tissues and the bone marrow. An “effective amount” is an amount of a mobilizing agent sufficient to effect a significant increase in the number and/or frequency of stem cells in the peripheral blood. An effective amount can be administered in one or more administrations, applications or dosages. A therapeutically effective amount of a mobilizing agent depends on the mobilizing agent selected, e.g., G-CSF or GM-CSF. The mobilizing agent, such as G-CSF or GM-CSF, can be administered from one or more times per day to one or more times per week; including once every other day. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to, previous treatments, the general health and/or age of the subject, and whether other diseases are present. Moreover, treatment of a subject with a therapeutically effective amount of the G-CSF or GM-CSF, as commonly known by persons of ordinary skill in the art, can include a single treatment or a series of treatments.

[00119] The term “mobilization” as used herein refers to the process whereby the cells leave the bone marrow and enter the blood. Mobilization may be effectuated by a combination of chemoattractants (e.g., cytokines) and loss of adhesiveness of pools or populations of stem cells residing in stem cell niches in peripheral tissues and the bone marrow.

[00120] As used herein, the term “stem cells” is used in a broad sense and includes traditional stem cells, progenitor cells, preprogenitor cells, reserve cells, and the like. The term “stem cell” or “progenitor” are used interchangeably herein, and refer to an undifferentiated cell which is capable of proliferation and of giving rise to more progenitor cells having the ability to generate a large number of mother cells that can in turn give rise to differentiated, or differentiable, daughter cells. The daughter cells themselves can be induced to proliferate and produce progeny that subsequently differentiate into one or more mature cell types, while also retaining one or more cells with parental developmental potential. The term “stem cell” refers then, to a cell with the capacity or potential, under particular circumstances, to differentiate to a more specialized or differentiated phenotype, and which retains the capacity, under certain circumstances, to proliferate without substantially differentiating. In one embodiment, the term progenitor or stem cell refers to a generalized mother cell whose descendants (progeny) specialize, often in different directions, by differentiation, e.g., by acquiring completely individual characters, as occurs in progressive diversification of embryonic cells and tissues. Cellular differentiation is a complex process typically occurring through many cell divisions. A differentiated cell may derive from a multipotent cell which

itself is derived from a multipotent cell, and so on. While each of these multipotent cells may be considered stem cells, the range of cell types each can give rise to may vary considerably. Some differentiated cells also have the capacity to give rise to cells of greater developmental potential. Such capacity may be natural or may be induced artificially upon treatment with various factors. In many biological instances, stem cells are also “multipotent” because they can produce progeny of more than one distinct cell type, but this is not required for “stemness.” Self-renewal is the other classical part of the stem cell definition, and it is essential as used in this document. In theory, self-renewal can occur by either of two major mechanisms. Stem cells may divide asymmetrically, with one daughter retaining the stem state and the other daughter expressing some distinct other specific function and phenotype. Alternatively, some of the stem cells in a population can divide symmetrically into two stems, thus maintaining some stem cells in the population as a whole, while other cells in the population give rise to differentiated progeny only. Formally, it is possible that cells that begin as stem cells might proceed toward a differentiated phenotype, but then “reverse” and re-express the stem cell phenotype, a term often referred to as “dedifferentiation.”

[00121] Exemplary stem cells include embryonic stem cells, adult stem cells, pluripotent stem cells, neural stem cells, liver stem cells, muscle stem cells, muscle precursor stem cells, endothelial progenitor cells, bone marrow stem cells, chondrogenic stem cells, lymphoid stem cells, mesenchymal stem cells, hematopoietic stem cells, central nervous system stem cells, peripheral nervous system stem cells, and the like. Descriptions of stem cells, including method for isolating and culturing them, may be found in, among other places, Embryonic Stem Cells, Methods and Protocols, Turksen, ed., Humana Press, 2002; Weisman et al., Annu. Rev. Cell. Dev. Biol. 17:387 403; Pittinger et al., Science, 284:143 47, 1999; Animal Cell Culture, Masters, ed., Oxford University Press, 2000; Jackson et al., PNAS 96(25):14482 86, 1999; Zuk et al., Tissue Engineering, 7:211 228, 2001 (“Zuk et al.”); Atala et al., particularly Chapters 33 41; and U.S. Pat. Nos. 5,559,022, 5,672,346 and 5,827,735. Descriptions of stromal cells, including methods for isolating them, may be found in, among other places, Prockop, Science, 276:71 74, 1997; Theise et al., Hepatology, 31:235 40, 2000; Current Protocols in Cell Biology, Bonifacino et al., eds., John Wiley & Sons, 2000 (including updates through March, 2002); and U.S. Pat. No. 4,963,489.

[00122] The term “progenitor cell” is used herein to refer to a stem cell that has a cellular phenotype that is more primitive (e.g., is at an earlier step along a developmental pathway or progression than is a fully differentiated cell) relative to a cell which it can give rise to by differentiation. Often, progenitor cells also have significant or very high proliferative

potential. Progenitor cells can give rise to multiple distinct differentiated cell types or to a single differentiated cell type, depending on the developmental pathway and on the environment in which the cells develop and differentiate.

[00123] As indicated above, there are different levels or classes of cells falling under the general definition of a “stem cell.” These are “totipotent,” “pluripotent” and “multipotent” stem cells. The term “totipotent” refers to a stem cell that can give rise to any tissue or cell type in the body. “Pluripotent” stem cells can give rise to any type of cell in the body except germ line cells. Stem cells that can give rise to a smaller or limited number of different cell types are generally termed “multipotent.” Thus, totipotent cells differentiate into pluripotent cells that can give rise to most, but not all, of the tissues necessary for fetal development. Pluripotent cells undergo further differentiation into multipotent cells that are committed to give rise to cells that have a particular function. For example, multipotent hematopoietic stem cells give rise to the red blood cells, white blood cells and platelets in the blood.

[00124] The term “pluripotent” as used herein refers to a cell with the capacity, under different conditions, to differentiate into cell types characteristic of all three germ cell layers (endoderm, mesoderm and ectoderm). Pluripotent cells are characterized primarily by their ability to differentiate to all three germ layers, using, for example, a nude mouse teratoma formation assay. Pluripotency is also evidenced by the expression of embryonic stem (ES) cell markers, although the preferred test for pluripotency is the demonstration of the capacity to differentiate into cells of each of the three germ layers. In some embodiments, a pluripotent cell is an undifferentiated cell.

[00125] The terms “pluripotency” or a “pluripotent state” as used herein refer to a cell with the ability to differentiate into all three embryonic germ layers: endoderm (gut tissue), mesoderm (including blood, muscle, and vessels), and ectoderm (such as skin and nerve), and that typically has the potential to divide in vitro for a long period of time, e.g., greater than one year or more than 30 passages.

[00126] The term “multipotent” when used in reference to a “multipotent cell” refers to a cell that is able to differentiate into some but not all of the cells derived from all three germ layers. Thus, a multipotent cell is a partially differentiated cell. Multipotent cells are well known in the art, and examples of multipotent cells include adult stem cells, such as for example, hematopoietic stem cells and neural stem cells. Multipotent means a stem cell may form many types of cells in a given lineage, but not cells of other lineages. For example, a multipotent blood stem cell can form the many different types of blood cells (red, white, platelets, etc.), but it cannot form neurons.

[00127] The term “multipotency” refers to a cell with the degree of developmental versatility that is less than totipotent and pluripotent.

[00128] The term “totipotency” refers to a cell with the degree of differentiation describing a capacity to make all of the cells in the adult body as well as the extra-embryonic tissues including the placenta. The fertilized egg (zygote) is totipotent as are the early cleaved cells (blastomeres).

[00129] The term “mesenchymal stem cell” is also referred to herein as “MSC” and refers to pluripotent stem cells capable of differentiating into more than one specific type of mesenchymal or connective tissue (i.e., tissues of the body which support specialized elements; e.g., adipose, osseous, stroma, cartilaginous, elastic and fibrous connective tissues). Human mesenchymal stem cells (hMSCs) are reactive with certain monoclonal antibodies, known as SH2, SH3 and SH4. (See U.S. Pat. No. 5,486,359, which is incorporated herein in its entirety by reference). MSCs can be differentiated from HSCs based on their immunospecific profiles and, with MSCs being SH2⁺/CD14⁻ and human HSCs SH2⁻/CD14⁺. For purposes of identification, human MSCs can be identified based on (i) phenotypic marker expression of CD34⁻, CD45⁻, CD90⁺, CD105⁺ and CD44⁺, (ii) functional phenotype, including the ability to form colony forming units in a CFA assay as disclosed in the Examples herein, and ability to differentiate into tissues which support specialized elements, including but not limited to: chondrocytes, cartilage and adipocytes. Other markers expressed by MSCs are known in the art and include without limitation CD71, CD73, Stro-1, and CD166, and CD271. In certain embodiments, MSCs are lin⁻.

[00130] The term “very small embryonic-like stem cell” is also referred to herein as “VSEL stem cell” and refers to pluripotent stem cells. In some embodiments, the VSEL stem cells (“VSELs”) are human VSELs and may be characterized as lin⁻, CD45⁻, and CD34⁺. In some embodiments, the VSELs are human VSELs and may be characterized as lin⁻, CD45⁻, and CD133⁺. In some embodiments, the VSELs are human VSELs and may be characterized as lin⁻, CD45⁻, and CXCR4⁺. In some embodiments, the VSELs are human VSELs and may be characterized as lin⁻, CD45⁻, CXCR4⁺, CD133⁺, and CD34⁺. Human VSELs express at least one of SSEA-4, Oct-4, Rex-1, and Nanog, and possess large nuclei surrounded by a narrow rim of cytoplasm, and contain embryonic-type unorganized chromatin. VSELs also have high telomerase activity. In some embodiments, the VSELs are human VSELs and may be characterized as lin⁻, CD45⁻, CXCR4⁺, CD133⁺, Oct 4⁺, SSEA4⁺, and CD34⁺. In some embodiments, the human VSELs may be less primitive and may be characterized as lin⁻, CD45⁻, CXCR4⁺, CD133⁻, and CD34⁺. In some embodiments, the human VSELs may be

enriched for pluripotent embryonic transcription factors, e.g., Oct-4, Sox2, and Nanog. In some embodiments, the human VSELs may have a diameter of 4-5 μm , 4-6 μm , 4-7 μm , 5-6 μm , 5-8 μm , 6-9 μm , or 7-10 μm .

[00131] The term “peripheral blood-derived” used in connection with a stem cell refers to a stem cell which is mobilized from the peripheral blood only, and can include expansion or proliferation of the stem cell in the peripheral blood. In some embodiments, the number of circulating stem cells can be increased in the peripheral blood by contacting the peripheral blood with a mobilizing agent, either *in vivo* or *ex vivo*, according to the methods as disclosed herein.

[00132] The term “bone marrow-derived” used in connection with a stem cell refers to a stem cell which is mobilized from the bone marrow to the peripheral blood, and can include stem cells which have migrated from the BM. In some embodiments, BM-derived stem cells in the peripheral blood includes stem cells which have proliferated in the bone marrow prior to migration to the peripheral blood, or alternatively stem cells which have proliferated in the peripheral blood after migration from the bone marrow. In some embodiments, the number of circulating BM-stem cells can be increased in the peripheral blood by contacting the peripheral blood with a mobilizing agent *in vivo* according to the methods as disclosed herein.

[00133] The term “hematopoietic stem cells,” also referred to as “HSCs,” refers to all stem cells or progenitor cells found *inter alia* in bone marrow and peripheral blood that are capable of differentiating into any of the specific types of hematopoietic or blood cells, such as erythrocytes, lymphocytes, macrophages and megakaryocytes. HSCs are reactive with certain monoclonal antibodies which are now recognized as being specific for hematopoietic cells, for example, monoclonal antibodies that recognize CD34.

[00134] The term “hematopoietic cells” refers to all types of hematopoietic cells throughout their differentiation from self-renewing hematopoietic stem cells through immature precursor cells of the various blood lineages, including the mature functioning blood cells, as would be understood by persons skilled in the art.

[00135] The terms “mesenchymal cell” or “mesenchyme” are used interchangeably herein and refer in some instances to the fusiform or stellate cells found between the ectoderm and endoderm of young embryos. Most mesenchymal cells are derived from established mesodermal layers but in the cephalic region they also develop from neural crest or neural tube ectoderm. Mesenchymal cells have a pluripotential capacity, particularly embryonic mesenchymal cells in the embryonic body, developing at different locations into any of the

types of connective or supporting tissues, to smooth muscle, to vascular endothelium, and to blood cells.

[00136] The term “isolated cell” as used herein refers to a cell that has been removed from a subject in which it was originally found or a descendant of such a cell. Optionally, the cell has been cultured *in vitro*, e.g., in the presence of other cells. Optionally, the cell, e.g., an isolated target stem cell population produced by the method as disclosed herein, is later introduced into a second subject or re-introduced into the subject from which it (or the cell from which it is descended) was isolated (e.g., allogenic transplantation).

[00137] The term “isolated population,” with respect to an isolated population of cells as used herein, refers to a population of cells that has been removed and separated from a mixed or heterogeneous population of cells. In some embodiments, an isolated population is a substantially pure population of cells as compared to the heterogeneous population from which the cells were isolated or enriched from. In some embodiments, the isolated population is an isolated population of reprogrammed cells which is a substantially pure population of reprogrammed cells as compared to a heterogeneous population of cells comprising reprogrammed cells and cells from which the reprogrammed cells were derived.

[00138] The term “substantially pure,” with respect to a particular target stem cell population, refers to a population of cells that is at least about 75%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% pure, with respect to the cells making up a total stem cell population. Recast, the terms “substantially pure” or “essentially purified,” with regard to a target stem cell population isolated using the methods as disclosed herein, refer to a population of target stem cells that contains fewer than about 20%, more preferably fewer than about 15%, 10%, 8%, 7%, most preferably fewer than about 5%, 4%, 3%, 2%, 1%, or less than 1%, of cells that are a non-target stem cell population as defined by the terms herein. In some embodiments, the present invention encompasses methods to expand a target stem cell population, wherein the expanded population of target stem cells is a substantially pure population of target stem cells.

[00139] As used herein, “proliferating” and “proliferation” refer to an increase in the number of cells in a population (growth) by means of cell division. Cell proliferation is generally understood to result from the coordinated activation of multiple signal transduction pathways in response to the environment, including growth factors and other mitogens. Cell proliferation may also be promoted by release from the actions of intra- or extracellular signals and mechanisms that block or negatively affect cell proliferation.

[00140] The term “regeneration” means regrowth of a cell population, organ or tissue after disease

or trauma.

[00141] The terms “renewal” or “self-renewal” or “proliferation” are used interchangeably herein, and refer to a process of a cell making more copies of itself (e.g., duplication of the cell). In some embodiments, reprogrammed cells are capable of renewal of themselves by dividing into the same undifferentiated cells (e.g., pluripotent or non-specialized cell type) over long periods, and/or many months to years. In some instances, proliferation refers to the expansion of reprogrammed cells by the repeated division of single cells into two identical daughter cells.

[00142] The term “lineages” as used herein refers to cells with a common ancestry or cells with a common developmental fate, for example, cells that are derived from the same target stem cell population or progeny thereof.

[00143] As used herein, the term “clonal cell line” refers to a cell lineage that can be maintained in culture and has the potential to propagate indefinitely. A clonal cell line can be a stem cell line (e.g., a target stem cell population cell line) or be derived from a target stem cell population, and where the clonal cell line is used in the context of a clonal cell line comprising a target stem cell population, the term refers to a target stem cell population which has been cultured under *in vitro* conditions that allow proliferation without differentiation for months to years. Such clonal stem cell lines (e.g., a target stem cell population) can have the potential to differentiate along several lineages of the cells from the original stem cell.

[00144] In the context of cell ontogeny, the adjective “differentiated,” or “differentiating” is a relative term. A “differentiated cell” is a cell that has progressed further down the developmental pathway than the cell it is being compared with. Thus, stem cells can differentiate to lineage-restricted precursor cells (such as a mesodermal stem cell), which in turn can differentiate into other types of precursor cells further down the pathway (such as an cardiomyocyte precursor), and then to an end-stage differentiated cell, which plays a characteristic role in a certain tissue type, and may or may not retain the capacity to proliferate further.

[00145] The term “differentiation” in the present context means the formation of cells expressing markers known to be associated with cells that are more specialized and closer to becoming terminally differentiated cells incapable of further differentiation. The pathway along which cells progress from a less committed cell to a cell that is increasingly committed to a particular cell type, and eventually to a terminally differentiated cell, is referred to as progressive differentiation or progressive commitment. Cells which are more specialized

(e.g., have begun to progress along a path of progressive differentiation) but not yet terminally differentiated are referred to as partially differentiated. Differentiation is a developmental process whereby cells assume a specialized phenotype, e.g., acquire one or more characteristics or functions distinct from other cell types. In some cases, the differentiated phenotype refers to a cell phenotype that is at the mature endpoint in some developmental pathway (a so called terminally differentiated cell). In many, but not all tissues, the process of differentiation is coupled with exit from the cell cycle. In these cases, the terminally differentiated cells lose or greatly restrict their capacity to proliferate. However, we note that in the context of this specification, the terms “differentiation” or “differentiated” refer to cells that are more specialized in their fate or function than at a previous point in their development, and includes both cells that are terminally differentiated and cells that, although not terminally differentiated, are more specialized than at a previous point in their development. The development of a cell from an uncommitted cell (for example, a stem cell) to a cell with an increasing degree of commitment to a particular differentiated cell type, and finally to a terminally differentiated cell, is known as progressive differentiation or progressive commitment. A cell that is “differentiated” relative to a progenitor cell has one or more phenotypic differences relative to that progenitor cell. Phenotypic differences include, but are not limited to, morphologic differences and differences in gene expression and biological activity, including not only the presence or absence of an expressed marker, but also differences in the amount of a marker and differences in the co-expression patterns of a set of markers.

[00146] The term “differentiation” as used herein refers to the cellular development of a cell from a primitive stage towards a more mature (i.e., less primitive) cell.

[00147] The term “directed differentiation” as used herein refers to forcing differentiation of a cell from an undifferentiated (e.g., more primitive cell) to a more mature cell type (i.e., less primitive cell) via genetic and/or environmental manipulation. In some embodiments, a reprogrammed cell as disclosed herein is subject to directed differentiation into specific cell types, such as neuronal cell types, muscle cell types and the like.

[00148] The term “media” as referred to herein is a medium for maintaining a tissue or cell population, or culturing a cell population (e.g., “culture media”) containing nutrients that maintain cell viability and support proliferation. The cell culture medium may contain any of the following in an appropriate combination: salt(s), buffer(s), amino acids, glucose or other sugar(s), antibiotics, serum or serum replacement, and other components such as peptide growth factors, etc. Cell culture media ordinarily used for particular cell types are known to

those skilled in the art.

[00149] The term “phenotype” refers to one or a number of total biological characteristics that define the cell or organism under a particular set of environmental conditions and factors, regardless of the actual genotype.

[00150] A “marker” as used herein describes the characteristics and/or phenotype of a cell.

Markers can be used for selection of cells comprising characteristics of interest. Markers will vary with specific cells. Markers are characteristics, whether morphological, functional or biochemical (enzymatic) characteristics particular to a cell type, or molecules expressed by the cell type. Preferably, such markers are proteins, and more preferably, possess an epitope for antibodies or other binding molecules available in the art. However, a marker may consist of any molecule found in a cell including, but not limited to, proteins (peptides and polypeptides), lipids, polysaccharides, nucleic acids and steroids. Examples of morphological characteristics or traits include, but are not limited to, shape, size, and nuclear to cytoplasmic ratio. Examples of functional characteristics or traits include, but are not limited to, the ability to adhere to particular substrates, ability to incorporate or exclude particular dyes, ability to migrate under particular conditions, and the ability to differentiate along particular lineages. Markers may be detected by any method available to one of skill in the art.

[00151] The term “contacting” or “contact” as used herein, as in connection with contacting a peripheral blood sample *in vivo*, can comprise administering a mobilizing agent, optionally in a composition, to a subject via an appropriate administration route such that the compound contacts the peripheral blood sample *in vivo*. The term “contacting” or “contact” as used herein, as in connection with contacting a peripheral blood sample *ex vivo*, can comprise administering a mobilizing agent, optionally in a composition, to a peripheral blood sample such that the mobilizing agent contacts the peripheral blood sample *ex vivo*.

[00152] As used herein, the terms “administering,” “introducing” and “transplanting” are used interchangeably and refer to the placement of a target stem cell population as described herein into a subject by a method or route which results in at least partial localization of the human target stem cell population at a desired site. The human target stem cell population can be administered by any appropriate route which results in delivery to a desired location in the subject where at least a portion of the human target stem cell population remain viable. The period of viability of the cells after administration to a subject can be as short as a few hours, e.g., twenty-four hours, to a few days, to as long as several years.

[00153] As used herein, the term “donor” refers to a subject from which an organ, tissue or cell to be transplanted is harvested from.

[00154] As used herein, the term “recipient” refers to a subject which will receive a transplanted organ, tissue or cell.

[00155] The term “graft” as used herein refers to the process whereby a free (unattached) cell, tissue, or organ integrates into a tissue following transplantation into a subject.

[00156] The term “allograft” refers to a transplanted cell, tissue, or organ derived from a different animal of the same species.

[00157] The terms “xenograft” or “xenotransplant” as used herein refer to a transplanted cell, tissue, or organ derived from an animal of a different species. In some embodiments, a xenograft is a surgical graft of tissue from one species to an unlike species, genus or family. By way of example, a graft from a baboon to a human is a xenograft.

[00158] The term “xenotransplantation” refers to the process of transplantation of living cells, tissues or organs from one species to another, such as from pigs to humans.

[00159] The terms “subject” and “individual” are used interchangeably herein, and refer to an animal, for example, a human, from whom a target stem cell population as disclosed herein can be isolated and collected according to the methods and compositions described herein, and optionally, a subject can receive a transplantation (e.g., the target stem cell population can be implanted into a subject), for example, for the treatment, including prophylactic treatment, of a disease. For treatment of disease states which are specific for a specific animal such as a human subject, the term “subject” refers to that specific animal. The terms “non-human animals” and “non-human mammals” are used interchangeably herein, and include mammals such as rats, mice, rabbits, sheep, cats, dogs, cows, pigs, and non-human primates. The term “subject” also encompasses any vertebrate including but not limited to mammals, reptiles, amphibians and fish. However, advantageously, the subject is a mammal such as a human, or other mammals such as a domesticated mammal, e.g., dog, cat, horse, and the like, or a production mammal, e.g., cow, sheep, pig, and the like.

[00160] The term “tissue” refers to a group or layer of similarly specialized cells which together perform certain special functions. The term “tissue-specific” refers to a source or defining characteristic of cells from a specific tissue.

[00161] The term “agent” as used herein means any compound or substance such as, but not limited to, a small molecule, nucleic acid, polypeptide, peptide, drug, ion, etc. An “agent” can be any chemical, entity or moiety, including, without limitation, synthetic and naturally-occurring proteinaceous and non-proteinaceous entities. In some embodiments, an agent is nucleic acid, nucleic acid analogues, proteins, antibodies, peptides, aptamers, oligomer of nucleic acids, amino acids, or carbohydrates, including, without limitation, oligonucleotides,

ribozymes, DNazymes, glycoproteins, siRNAs, lipoproteins, and modifications and combinations thereof etc. In certain embodiments, agents are small molecule having a chemical moiety. For example, chemical moieties included unsubstituted or substituted alkyl, aromatic, or heterocyclyl moieties, including macrolides, leptomycins and related natural products or analogues thereof. Compounds can be known to have a desired activity and/or property, or can be selected from a library of diverse compounds.

[00162] As used herein, the term “small molecule” refers to a chemical agent which can include, but is not limited to, a peptide, a peptidomimetic, an amino acid, an amino acid analog, a polynucleotide, a polynucleotide analog, an aptamer, a nucleotide, a nucleotide analog, an organic or inorganic compound (e.g., including heterorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

[00163] The term “disease” or “disorder” is used interchangeably herein, and refers to any alternation in state of the body or of some of the organs, interrupting or disturbing the performance of the functions and/or causing symptoms such as discomfort, dysfunction, distress, or even death to the person afflicted or those in contact with the person. A disease or disorder can also relate to a distemper, ailing, ailment, malady, disorder, sickness, illness, complaint, indisposition or affection.

[00164] The term “pathology” as used herein, refers to symptoms, for example, structural and functional changes in a cell, tissue, or organs, which contribute to a disease or disorder. For example, the pathology may be associated with a particular nucleic acid sequence, or “pathological nucleic acid” which refers to a nucleic acid sequence that contributes, wholly or in part to the pathology, as an example, the pathological nucleic acid may be a nucleic acid sequence encoding a gene with a particular pathology causing or pathology-associated mutation or polymorphism. The pathology may be associated with the expression of a pathological protein or pathological polypeptide that contributes, wholly or in part, to the pathology associated with a particular disease or disorder. In another embodiment, the pathology is, for example, associated with other factors, for example ischemia and the like.

[00165] As used herein, the term “treating” includes reducing or alleviating at least one adverse effect or symptom of a condition, disease or disorder.

[00166] The phrases “parenteral administration” and “administered parenterally” as used herein

mean modes of administration other than enteral and topical administration, usually by injection, and include, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intraventricular, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, sub capsular, subarachnoid, intraspinal, intracerebro spinal, and intrasternal injection and infusion. The phrases “systemic administration,” “administered systemically,” “peripheral administration” and “administered peripherally” as used herein mean the administration of a target stem cell population or differentiated progeny thereof and/or their progeny and/or compound and/or other material other than directly into the subject, such that it enters the animal’s system and, thus, is subject to metabolism and other like processes, for example, subcutaneous or intravenous administration.

[00167] The phrase “pharmaceutically acceptable” is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

[00168] The phrase “pharmaceutically acceptable carrier” as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject agents from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be “acceptable” in the sense of being compatible with the other ingredients of the formulation.

[00169] The term “drug” or “compound” as used herein refers to a chemical entity or biological product, or combination of chemical entities or biological products, administered to a subject to treat or prevent or control a disease or condition. The chemical entity or biological product is preferably, but not necessarily a low molecular weight compound, but may also be a larger compound, for example, an oligomer of nucleic acids, amino acids, or carbohydrates including without limitation proteins, oligonucleotides, ribozymes, DNazymes, glycoproteins, siRNAs, lipoproteins, aptamers, and modifications and combinations thereof.

[00170] The term “transplantation” as used herein refers to introduction of new cells (e.g., a target stem cell population or differentiated progeny thereof), or tissues (such as differentiated cells produced from a target stem cell population), or organs into a host (i.e., transplant recipient or transplant subject).

[00171] The term “modulate” is used consistently with its use in the art, e.g., meaning to cause or facilitate a qualitative or quantitative change, alteration, or modification in a process, pathway, or phenomenon of interest. Without limitation, such change may be an increase, decrease, or change in relative strength or activity of different components or branches of the process, pathway, or phenomenon. A “modulator” is an agent that causes or facilitates a qualitative or quantitative change, alteration, or modification in a process, pathway, or phenomenon of interest.

[00172] The terms “decrease,” “reduced,” “reduction,” “decrease” or “inhibit” are all used herein generally to mean a decrease by a statistically significant amount. However, for avoidance of doubt, “reduced,” “reduction” or “decrease” or “inhibit” means a decrease by at least 10% as compared to a reference level, for example a decrease by at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% decrease (e.g., absent level as compared to a reference sample), or any decrease between 10-100% as compared to a reference level.

[00173] The terms “increased,” “increase” or “enhance” or “activate” are all used herein to generally mean an increase by a statically significant amount, for the avoidance of any doubt, the terms “increased,” “increase” or “enhance” or “activate” means an increase of at least 10% as compared to a reference level, for example an increase of at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90% or up to and including a 100% increase or any increase between 10-100% as compared to a reference level, or at least about a 2-fold, or at least about a 3-fold, or at least about a 4-fold, or at least about a 5-fold or at least about a 10-fold increase, or any increase between 2-fold and 10-fold or greater as compared to a reference level.

[00174] The term “statistically significant” or “significantly” refers to statistical significance and generally means a two standard deviation (2SD) below normal, or lower, concentration of the marker. The term refers to statistical evidence that there is a difference. It is defined as the probability of making a decision to reject the null hypothesis when the null hypothesis is actually true. The decision is often made using the p-value. The term “substantially” as used herein means a proportion of at least about 60%, or preferably at least about 70%, or at least about 80%, or at least about 90%, at least about 95%, at least about 97%, or at least about 99% or more, or any integer between 70% and 100%.

[00175] As used in this specification and the appended claims, the singular forms “a,” “an,” and “the” include plural references unless the context clearly dictates otherwise. Thus for example, references to “the method” includes one or more methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[00176] It is understood that the foregoing detailed description and the following examples are illustrative only and are not to be taken as limitations upon the scope of the invention. Various changes and modifications to the disclosed embodiments, which will be apparent to those of skill in the art, may be made without departing from the spirit and scope of the present invention. Further, all patents, patent applications, and publications identified are expressly incorporated herein by reference for the purpose of describing and disclosing, for example, the methodologies described in such publications that might be used in connection with the present invention. These publications are provided solely for their disclosure prior to the filing date of the present application. Nothing in this regard should be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or for any other reason. All statements as to the date or representation as to the contents of these documents are based on the information available to the applicants and do not constitute any admission as to the correctness of the dates or contents of these documents.

Elutriation devices:

[00177] One aspect of the present invention is to separate different target stem cell populations from the peripheral blood into subpopulations of stem cells using centrifugal elutriation. Without wishing to be bound by theory, in one method of elutriation, a peripheral blood sample is introduced into a generally funnel-shaped separation chamber located on a spinning centrifuge. A flow of liquid elutriation buffer or low density liquid is then introduced into the chamber containing the peripheral blood sample. As the flow rate of the liquid elutriation buffer solution is increased through the chamber (usually in a stepwise manner), the liquid sweeps smaller sized, slower-sedimenting cells toward an elutriation boundary within the chamber, while larger, faster-sedimenting cells migrate to an area of the chamber where the centrifugal force and the sedimentation (drag) forces are balanced. Accordingly, as the peripheral blood, e.g., mobilized peripheral blood, comprises many different populations of stem cells, one aspect of the present invention relates to the separation of these different stem cell populations present in peripheral blood into distinct populations using elutriation, where smaller stem cells are fractionated from stem cells which are larger in size.

[00178] In some aspects of the present invention, any elutriation device can be used to fractionate a peripheral blood sample into fractions comprising different stem cell populations. In some embodiments, commercially available elutriation devices can be used, for example, the ELUTRA® centrifuge manufactured by Gambro BCT, Inc. The ELUTRA CELL SEPARATION SYSTEM® enables the separation of cell populations into multiple fractions based on both size and density, enabling cell enrichment, depletion, concentration, and washing all within a functionally-closed system. The ELUTRA CELL SEPARATION SYSTEM® enables enrichment of stem cell populations directly from leukapheresis products without antibodies or preprocessing in less than one hour. The ELUTRA CELL SEPARATION SYSTEM® uses counterflow centrifugal elutriation, where fluid flows through cell layers in a centrifugal field in order to separate cell populations.

[00179] In some embodiments, other elutriation devices can be used, including but not limited to, the COBE® Spectra apheresis system, the TRIMA® system and the TRIMA ACCEL® system, also manufactured by Gambro BTC Inc., as well as other commercially available elutriation devices used to separate blood components.

[00180] In some embodiments, the peripheral blood is fractionated using a cell separator such as the COBE® Spectra Apheresis System. The COBE® SPECTRA™ centrifuge is described in U.S. Patents 4,425,172; 4,708,712; and 6,022,306, which are incorporated herein by reference. In such an embodiment, the peripheral blood sample is drawn into a cell separator such as the COBE® Spectra Apheresis System, and, optionally, an anticoagulant solution is added to the blood to keep it from clotting during the procedure. The blood/anticoagulant admixture cycles through a centrifuge to separate the peripheral blood sample into stem cell populations and mononuclear cells from the other blood components and plasma. The system pumps the separated stem cells into a collection bag for storage, while the other blood components and plasma return to the patient. All tubing sets and needles used are sterile, so there is no risk of disease transmission.

[00181] Other blood-separation apparatuses, such as the apparatus described in U.S. Pat. No. 5,722,926, issued Mar. 3, 1998; U.S. Pat. No. 5,951,877, issued Sep. 14, 1999; U.S. Pat. No. 6,053,856, issued Apr. 25, 2000; U.S. Pat. No. 6,334,842, issued Jan. 1, 2002; U.S. patent application Ser. No. 10/884,877 filed Jul. 1, 2004; U.S. Pat. 7,201,848; U.S. Patent 6,022,306; U.S. Patent 6,589,526; U.S. Patent Applications 2008/0035585; US2008/0318756 and 2009/0104626 can be used. The entire disclosure of each of these U.S. patents and patent applications is incorporated herein by reference.

[00182] Without being bound by theory, leukapheresis refers to one type of separation procedure which separates white blood cells from the other particles, and typically uses a centrifuge. The resulting harvested or separated white blood cells can then be further separated into subsets of desired cells (e.g., monocytes, lymphocytes, and granulocytes) for collection or culture, if desired, although it is understood that collection of other cells may also be desired. The collected leukapheresis products, however, are often contaminated with platelets and red blood cells which can interfere with various cell separation and/or cell selection techniques and later cultivation of the selected cells for therapeutic use. Thus, it is desirable to separate the white blood cells into the desired subsets and remove or reduce the number of platelets and red blood cells.

[00183] Several methods have been proposed for the separation or fractionation of white blood cells from other particles and into more purified selected subsets. One such method is centrifugal elutriation. In one common form of elutriation, a cell batch is introduced into a generally funnel-shaped separation chamber located on a spinning centrifuge. A flow of liquid elutriation buffer or low density liquid is then introduced into the chamber containing the cell batch. As the flow rate of the liquid elutriation buffer solution is increased through the chamber (usually in a stepwise manner), the liquid sweeps smaller sized, slower-sedimenting cells toward an elutriation boundary within the chamber, while larger, faster-sedimenting cells migrate to an area of the chamber where the centrifugal force and the sedimentation (drag) forces are balanced.

[00184] Relatively long elutriation times may be needed to achieve optimum separation and purification of the selected cells into subsets of interest. Such long time periods require a continuous flow of the liquid elutriation buffer into the chamber. This in turn may require a larger volume bag and/or multiple smaller bags to hold the amount of liquid or buffer needed for optimal cell separation. Also the use of larger volumes of elutriation buffer adds additional expense to each separation process.

[00185] Preferably, the elutriation method as used herein can separate the peripheral blood sample into various target stem cell populations which does not affect the function or viability of the stem cells.

[00186] Collection may be performed from peripheral blood obtained from any subject, such as a human subject, including an adult or a non-neonate child. Furthermore, collection may involve one or more collecting steps or collecting periods. For example, collection (e.g., using an apheresis process) may be performed at least two times, at least three times, or at

least 5 times, on a person. During each collecting step, the number of total nucleated cells collected per kilogram weight of the person may be one million (1×10^6) or more (e.g., 1×10^7 , 1×10^8 , 1×10^9 , 1×10^{10} , 1×10^{11} , 1×10^{12} , 1×10^{13} , 1×10^{14} , 1×10^{15} , 1×10^{16} , 1×10^{17} , 1×10^{18} , 1×10^{19} , 1×10^{20}). In preferred embodiments, the number of cells collected in a single collection session may be equal or greater than 1×10^{15} , total nucleated cells, or at least on the order of 1×10^{14} , 1×10^{13} , 1×10^{12} , 1×10^{11} , 1×10^{10} , 1×10^9 , 1×10^8 , 1×10^7 , 1×10^6 , 1×10^5 , total nucleated cells, depending on the weight and age of the donor subject.

[00187] Depending on the situation and the quantity and quality of stem cells to be collected from the human donor, it may be preferable to collect the stem cells from human subject donors when they are at an “adult” or a “matured” age (the term “adult” as used herein refers to and includes adult and non-neonate, unless otherwise used in a particular context to take a different meaning) and/or at a certain minimum weight. For example, stem cell populations can be collected when the subject is within a weight range from 10 to 200 kg in accordance with one embodiment of the present invention, or any range within such range, such as 20 to 40 kg. In addition or in the alternative, it may be required that the subject be of a certain age, within a range from 2-80 years old (e.g., 2-10, 10-15, 12-18, 16-20, 20-26, 26-30, 30-35, 30-40, 40-45, 40-50, 55-60, 60-65, 60-70, and 70-80 years old) in accordance with one embodiment of the present invention.

Flow rates:

[00188] In some embodiments, the rate of the flow of the liquid elutriation buffer determines the size of the target stem cells to be separated from the remaining stem cells. In some embodiments, a target stem cell population of small stem cells, for example, including, but not limited to, VSELs can be separated from the other stem cells in the peripheral blood when the elutriation flow rate is at least about 50ml/minute. In some embodiments, a target stem cell population of stem cells which are larger than the size of VSELs, can be separated from the other stem cells in the peripheral blood when the elutriation flow rate is at least about 70ml/minute. In some embodiments, a target stem cell population of stem cells can be separated from the other stem cells in the peripheral blood when the elutriation flow rate is at least about 90ml/minute. In some embodiments, a target stem cell population of stem cells can be separated from the other stem cells in the peripheral blood when the elutriation flow rate is at least about >90ml/minute, or about 105ml/minute.

[00189] In some embodiments, a flow rate of less than about 20ml/minute can be used to collect platelets from the peripheral blood. In some embodiments, a flow rate of between about 2ml/minute to about 20ml/minute can be used to collect platelets.

[00190] In some embodiments, the first fraction is collected at a flow rate of about 20ml/minute or less. In some embodiments, a second fraction is collected at a flow rate of between 20ml/min to about 50ml/min, such as about at least 20ml/minute, or at least 30ml/minute, or about 40ml/minute, or about 50ml/minute. In some embodiments, a third fraction is collected at a flow rate of between 51ml/min to about 70ml/min, such as about at least 51ml/minute, or at least 60ml/minute, or about 65ml/minute, or about 70ml/minute. In some embodiments, a fourth fraction is collected at a flow rate of between 71ml/minute to about 90ml/minute, such as about at least 71ml/minute, or at least 80ml/minute, or about 85ml/minute, or about 90ml/minute. In some embodiments, a fifth fraction is collected at a flow rate of between about 91ml/minute to about 105ml/minute or greater than 105ml/minute, such as about at least 91ml/minute, or at least 95ml/minute, or about 100ml/minute, or about 105ml/minute. Alternatively, in some embodiments, the fifth fraction can be collected as subfractions, for example cells can be collected at one or more of the following flow rates; at least about 95ml/minute, or at least about 100ml/minute, or about 105ml/minute, or greater than 105ml/minute. Such an embodiment may be useful to collect subfractions comprising substantially different target stem cell populations, for example, where one subfraction of collected cells comprises substantially HSCs, and another subfraction of collected cells comprises substantially MSC.

[00191] In some embodiments, relatively long elutriation times may be needed to achieve optimum separation and purification of the selected stem cells into subsets of stem cells of interest (e.g., target stem cell populations). Such long time periods require a continuous flow of the liquid elutriation buffer into the chamber. This in turn may require a larger volume bag and/or multiple smaller bags to hold the amount of liquid or buffer needed for optimal cell separation. Also, the use of larger volumes of elutriation buffer adds additional expense to each separation process.

[00192] In some embodiments, the elutriation flow rate is set or other parameters are set to allow separated fractions to be continuously analyzed at the time of the separation process not only with respect to cell size but also in relation to other cell properties of importance, such as specific density, morphology, and cell surface markers, as disclosed in U.S. Patent No. 4,939,081, which is incorporated herein by reference.

[00193] The term “optimal setting” as used herein refers to the setting which leads to such fractionation that, in the cell population of a fraction, an optimal number of cells are present with a cell property of a particular value and lying within a particular range of values. For example, the optimal settings for fractionation of a peripheral blood sample, such as a mobilized peripheral blood sample, are shown in Table 1.

[00194] Table 1: optimal settings for separating different target stem cell populations from peripheral blood obtained from humans using an ELUTRA® apparatus operating at a centrifugal speed of 2400 rpm, corresponding to 825 x g.

Fraction	Optimal setting (flow rate)	Target cell population	Yield pre-elutriation	Yield post-elutriation	Yield increase (fold enrichment)
FRACTION 1	>20ml/min	Platelets			
FRACTION 2	50ml/min	VSELs	0.001%	0.3%	30-fold
FRACTION 3	70ml/min				
FRACTION 4	90ml/min	WBC & RBC			
FRACTION 5	>90ml/min	HSC & MSC			

[00195] Another suitable combination of flow rates for separating different target stem cell populations from peripheral blood obtained with humans using and an ELUTRA® apparatus operating at a centrifugal speed of 2400 rpm is as follows:

platelets	35 ml/min
VSELs	50 ml/min
VSELs	70 ml/min
Red blood cells, some HSCs	90 ml/min
pure HSCs	100 ml/min
MSCs	110 ml/min
MSCs	120 ml/min

[00196] One of ordinary skill in the art would be able to select flow rates suitable for collecting particular stem cell populations using elutriation devices other than the ELUTRA® based on the flow rates and centrifugation speeds disclosed herein that were used to collect those stem cell populations with the ELUTRA®. Using the flow rates and centrifugation speeds disclosed herein, as well as common knowledge in the art such as the Stoke’s equation

referred to below, it would be a routine matter to select appropriate flow rates to collect a desired stem cell population on another elutriation device.

[00197] Accordingly, in some embodiments the desired target stem cell population in a fraction may be less than 1% of the total number of cells in the fraction obtained by the elutriation separation process, e.g., as in the case of Fraction 2, where the desired target population is VSELs (Very Small Embryonic-like cells) which comprise 0.3% of the total cells in Fraction 2, but is a 30-fold enrichment as compared to the pre-elutriation peripheral blood sample.

[00198] In some embodiments, the second fraction (Fraction 2), or the fraction collected from a flow rate of about 50ml/minute, comprises at least about 0.05%, or at least about 0.1%, or at least about 0.2%, or about 0.3%, or at least about 0.4%, or greater than 0.4% of VSELs. In some embodiments, the second fraction (Fraction 2), or a fraction of cells collected from a flow rate of about 50ml/minute can be separated into different subpopulations of cells, for example, into a substantially pure population of VSELs. A substantially pure population of VSELs comprise at least about 75%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% VSELs, with respect to the cells making up a total cell population.

[00199] In some embodiments, the fifth fraction (Fraction 5), or the fraction collected from a flow rate of greater than 90ml/minute, such as about 105ml/minute or greater than 105ml/minute comprises at least about 1%, or at least about 2%, or at least about 3%, or about 4% or at least about 5%, or greater than 5% of a cells which are either HSCs or MSCs. In some embodiments, the fifth fraction (Fraction 5), or a fraction of cells collected from a flow rate of about 105ml/minute or greater than 105ml/minute can be further fractionated such that the population of HSCs and MSCs can be separated from non-HSC cells and non-MSC cells, and, further, the HSC cells can be separated from a population of MSCs. Accordingly, the fifth fraction (Fraction 5), or a fraction of cells collected from a flow rate of about >90ml/minute, such as 105ml/minute, or greater than 105ml/minute can be separated into subpopulations of cells, for example, into a substantially pure population of HSC cells and into a substantially pure population of MSC cells. A substantially pure population of HSCs comprises at least about 75%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% HSCs, with respect to the cells making up a total cell population. Similarly, a substantially pure population of MSCs comprise at least about 75%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% MSCs, with respect to the cells making up a total cell

population

[00200] It is encompassed in the present invention that the fractionation of the peripheral blood into fractions is not limited in number. Stated another way, the peripheral blood can be subjected to any number of different flow rates to yield any number of fractions. For example, the peripheral blood can be separated into at least 4, or at least 5, or at least 6, or at least 7, or at least 8, or at least 9, or at least 10, or at least 11, or at least 12, or more than 12 different fractions. For example, in some embodiments, the peripheral blood can be separated into a number multiple different fractions by subjecting the peripheral blood to at least 4, or at least 5, or at least 6, or at least 7, or at least 8, or at least 9, or at least 10, or at least 11, or at least 12, or more than 12 different flow rates.

[00201] In some embodiments, the desired yield of a target stem cell population is determined by the target collected volume as follows:

$$\frac{\text{yield of target stem cells}}{\text{concentration of target stem cells}} = \text{target collected volume for the target stem cell population}$$

[00202] The target flow rate can be calculated as discussed in Table 1 in U.S. Patent No. 6,022,306, which is incorporated herein by reference.

[00203] In some embodiments, the centrifugal elutriation separates cell particles having different sedimentation velocities, as disclosed in U.S. Patent No. 7,201,848, which is incorporated herein in its entirety by reference. Stoke's law describes sedimentation velocity (SV) of a spherical particle, as follows:

$$SV = \frac{2}{9} \frac{r^2(\rho_p - \rho_m)g}{\eta}$$

where,

[00204] r is the radius of the particle,

[00205] ρ_p is the density of the particle,

[00206] ρ_m is the density of the liquid medium,

[00207] η is the viscosity of the medium, and

[00208] g is the gravitational or centrifugal acceleration.

[00209] Because the radius of a particle is raised to the second power in the Stoke's equation and the density of the particle is not, the size of a cell, rather than its density, greatly influences its

sedimentation rate. This explains why larger particles generally remain in a chamber during centrifugal elutriation, while smaller particles are released, if the particles have similar densities.

[00210] As described in U.S. Patent No. 3,825,175, which is incorporated herein in its entirety by reference, centrifugal elutriation has a number of limitations. In most of these processes, particles must be introduced within a flow of fluid medium in separate, discontinuous batches to allow for sufficient particle separation. Thus, some elutriation processes only permit separation in particle batches and require an additional fluid medium to transport particles. In addition, flow forces must be precisely balanced against centrifugal force to allow for proper particle segregation. Thus, the present invention encompasses centrifugal elutriation as disclosed in U.S. Patent Application 2008/0318756, which is incorporated herein in its entirety by reference.

Peripheral blood sample for enriching subpopulations of stem cells by elutriation

[00211] In some embodiments, the peripheral blood sample which is used for the methods as disclosed herein is obtained from a mammalian subject, such as a human subject. In some embodiments, the human subject has been previously administered a mobilizing agent to increase the yield or number of circulating stem cells in the peripheral blood. Mobilization methods are known in the art, and include without limitation, methods as disclosed in U.S. Patent No. 6,261,549 and U.S. Patent Application 2009/0155225, which are incorporated herein by reference in their entirety.

[00212] In some embodiments, mobilizing agents administered to a subject include, but are not limited to, G-CSF, GM-CSF, dexamethasone, a CXCR4 receptors inhibitor, Interleukin-1 (IL-1), Interleukin-3 (IL-3), Interleukin-8 (IL-8), PIXY-321 (GM-CSF/IL-3 fusion protein), macrophage inflammatory protein, stem cell factor, thrombopoietin and growth related oncogene, as single mobilization agents or in any combination of such mobilizing agents. Other mobilizing agents include for example, but are not limited to CXCR4 inhibitors, such as AMD3100, ALX40-4C, T22, T134, T140 and TAK-779, which are disclosed in U.S. Patent No. 7,169,750, which is incorporated herein by reference in its entirety.

[00213] In some embodiments, the peripheral blood sample is a mobilized peripheral blood sample. In some embodiments, the peripheral blood is harvested or obtained from a subject after at least 2 days following administration of a mobilizing agent. In some embodiments,

the peripheral blood sample is harvested or obtained from a subject after 4 days or less following administration of an effective amount of a mobilizing agent to the subject.

[00214] In some embodiments, a peripheral blood sample or mobilized peripheral blood sample is pre-treated, e.g., by apheresis or some other method (e.g., chemical lysis of lymphocytes) prior to elutriation process to separate multiple target stem cell populations according to the methods as disclosed herein. Accordingly, in some embodiments, the starting material for elutriation according to the methods as disclosed herein is an apheresis product rather than the peripheral blood sample itself, where the apheresis product is processed from the peripheral blood sample.

[00215] The subjects amenable to collecting a variety of stem cell populations from peripheral blood using the methods as disclosed herein can be any subject, such as any human subject, at any age. In some embodiments, the subject is a child, such as from age of about 1 to 18 years of age. In some embodiments, the subject is an adult subject. In some embodiments, the subject is a healthy subject. In some embodiments, the subject has a predisposition to a disease (e.g., diabetes, cardiovascular disease, etc.), such as a subject having a mutation or genetic marker (such as a SNP, etc.) in a gene which is associated to an increase risk that the subject will likely develop the disease at some future date. In some embodiments, as discussed above, the subject is administered a mobilizing agent prior to obtaining a peripheral blood sample in the subject. In some embodiments, the subject is preferably at least 10 years old, or at least 18 years old, or at least about 30 years old. In some embodiments, the subject is preferably less than about 60 years of age, or less than about 50 years of age, or less than about 40 years of age, or less than about 30 years of age, or less than about 20 years of age.

[00216] The inventors have discovered that hematocrit ranges employed for apheresis can be advantageously adjusted to enhance properties of the collected cell population. For example, in one embodiment, the hematocrit range is selected to enhance the proportion of VSELs. In another embodiment, the hematocrit range is selected to enhance cryopreservation of collected VSELs, for example by minimizing collection of granulocytes. In another embodiment, the hematocrit range is selected to ensure simultaneous collection of multiple stem cell populations, such as, for example, VSELs and MSCs. In one embodiment, the hematocrit range is selected to be 2-3%. In another embodiment, the hematocrit range is selected to be 3-4%

Use of the enriched stem cell populations from peripheral blood

[00217] In some embodiments, the enriched populations of target stem cells are stored for future use in a stem cell bank, e.g., for use in the treatment of a disease or protocol for regenerative medicine or therapy for the subject of whom the cells were originally obtained (e.g., the cells are used in subsequent allogenic transplantation procedures). In such embodiments, the target stem cells obtained by the methods as disclosed herein are processed for moderate to long-term storage in a cell bank. This procedure has advantages in that different target stem cell populations can be stored such that the target stem cells population most suitable for the subsequent procedure can be selected for use in the regenerative therapy procedure.

Additionally, in some circumstances in tissue engineering and regenerative therapy, it may be desirable to use a combination, and in different ratios, of different target stem cell populations for different tissue engineering or regenerative therapy procedures. Thus, the present invention provides the ability to tailor the use of the target stem cell populations for future use.

[00218] In some embodiments, the different populations of stem cells obtained from peripheral blood by the methods as disclosed herein can be cryopreserved (e.g., frozen) at liquid nitrogen temperatures and stored for long periods of time, being capable of use on thawing, for example, for use in therapeutic purposes such as regenerative therapy or medicine. In some embodiments, the fractions obtained from the peripheral blood can be further separated into subpopulations of stem cells prior to cryopreservation. In some embodiments, the different target populations of stem cells obtained in each fraction can be further purified and expanded (e.g., propagated by *in vitro* culturing) to generate a substantially pure population of the target stem cells prior to being cryopreserved.

[00219] If frozen, the stem cell populations can be stored in a 10% DMSO, 50% FCS, 40% RPMI 1640 medium. Once thawed, the stem cell populations can be cultured and expanded *ex vivo* in the presence of growth factors and/or feeder layers for an appropriate period of time prior to implanting into a subject in need thereof.

[00220] Methods of cryopreservation of stem cells are commonly known by one of ordinary skill in the art and are disclosed in U.S. Patent Applications 2008/0220520, 2009/0022693, 2008/0241113 and 2005/0106554 and U.S. Patents 5,759,764 and 7,112,576 and 7,604,930, which are incorporated herein in their entirety by reference.

[00221] For cryopreservation, each population of stem cells obtained from peripheral blood by the methods as disclosed herein, can be suspended in DPBS and may be placed on ice for at least about 15 minutes in preparation for cryopreservation. The preparation may comprise adding cryopreservation media to the target stem cell population or a substantially pure population of

target stem cells and then subjecting the mixture to several temperature reduction steps to reduce the temperature of the target stem cell population or a substantially pure population of target stem cells to a final temperature of about -90° C., utilizing a controlled rate freezer or other suitable freezer system (dump-freeze monitored or a freeze container (Nalgene)).

Suitable control rate freezers include, but are not limited to, Cryomed Thermo Form a Controlled Rate Freezer 7454 (Thermo Electron, Corp.), Planar Controlled Rate Freezer Kryo 10/16 (TS Scientific), Gordinier, Bio-Cool--FTS Systems, and Asymptote EF600, BIOSTOR CBS 2100 series.

[00222] Cryopreservation media may be prepared comprising media and DMSO. About 3 ml of DPBS may be added to a container, such as, for example, a 50 ml conical tube. About 1 ml of human serum albumin (HSA) may be added to the about 3 ml of DPBS and then chilled for about ten minutes on ice. About 1 ml of the chilled 99% DMSO is added to the HSA and DPBS to prepare the final cryopreservation media. Cryopreservation media and the target stem cell population, or a substantially pure population of target stem cells, may then be placed on ice for about 15 minutes before the cryopreservation media is added to the cell sample. Batch processing may be used for aliquoting cryopreservation media into a cell sample. For example, a single aliquot of about 100 µl of the target stem cell population, or a substantially pure population of target stem cells, may be combined with about 3 ml of DPBS, 1 ml of HSA, and about 1 ml of 99% DMSO. About 2 aliquots of about 200 µl of MSC cell suspension may be combined with about 6 ml of DPBS, 2 ml of HSA, and about 2 ml of 99% DMSO. About 5 aliquots of cell sample may be combined with about 15 ml of DPBS, about 5 ml of HSA, and about 5 ml of 99% DMSO. About 10 aliquots of cell sample may be combined with about 30 ml of DPBS, about 10 ml of HSA, and about 10 ml of 99% DMSO.

[00223] In an alternative embodiment, other cryopreservation media may be used. For example, cryopreservation media with cryopreservation agents may be used to maintain a high cell viability outcome post-thaw, such as, for example, CryoStor CS10 or CS5 (Biolife), embryonic cryopreservation media supplemented with propanediol and sucrose (Vitrolife), or SAGE media (Cooper Surgical). Glycerol may be used with other cryopreservation agents, such as, DMSO, or may be used alone at a concentration of about 10% in a media with suitable protein.

[00224] In another embodiment, the cryopreservation media may be added to the target stem cell population, or a substantially pure population of target stem cells. Cryopreservation media

may be added to the target stem cell population, or a substantially pure population of target stem cells suspended in DPBS drop by drop until the volume of total mixture of suspended menstrual stem cells and cryopreservation media is brought to the final desired volume of cryopreservation cell mixture. The final cryopreservation cell media may be transferred into 2×5 ml bar-coded cryoquats with the QC sample stored in the cap of the 5 ml vial. Use a pipettor to remove about 200 µl aliquots of the sample and aliquot into the QC caps. After the caps have been filled, add the remaining 4.8 ml of specimen into the 5 ml vial. Samples should be sent for infectious disease and other analysis. The cryovials should be placed in the CRYOMED® Freezer and subject to temperature reduction by controlled rate to a temperature at or below about -85° C. The cryopreserved specimens should be transferred to a bunker for storage in the vapor of Liquid Nitrogen at -150° C. or less. Any sample that tests positive for an infectious disease should be quarantined. Any sample that tests negative for infectious disease may be transferred to a different permanent storage.

[00225] The following temperature reduction steps may be programmed in the controlled rate freezer, first reducing the mixture of cryopreservation agent and the target stem cell population, or a substantially pure population of target stem cells. The target stem cell population, or a substantially pure population of target stem cells combined with cryopreservation agent may be subjected to controlled rate temperature reductions in preparation for final storage in a freezer. The controlled rate reductions may be designed to maintain cell viability. A Cryo-Med Freezer (Thermo Electron Corp.), liquid nitrogen cylinder, and portable Cryo-Med Freezer may be used for controlled rate reductions in preparation for final storage in a freezer. The cells may be subject to controlled rate reductions in cryovials or cryobags to reach a temperature of about -90° C.

[00226] For a sample of the target stem cell population, or a substantially pure population of target stem cells collected in a cryobag, the cells may be subject to the following controlled rate reduction profile: wait at about 4° C., 1.0° C. per minute to -6.0° C. (sample), 25.0° C. per minute to -50.0° C. (chamber), 10.0° C. per minute to -14.0° C. (chamber), 1.0° C. per minute to -45.0° C. (chamber), 10.0° C. per minute to -90.0° C. (chamber), and end (sample at or below -85.0° C.).

[00227] For a sample of the target stem cell population, or a substantially pure population of target stem cells collected in a cryovial, the cells may be subject to the following controlled rate reduction profile: wait at 4.0° C., 1.0° C. per minute to -3.0° C. (chamber), 10.0° C. per minute to -20.0° C. (chamber), 1.0° C. per minute to -40.0° C. (chamber), 10.0° C. per minute to -90.0° C. (chamber), and end.

- [00228] Once the mixture of cryopreservation agent and the target stem cell population, or a substantially pure population of target stem cells, is at or below about -85° C., the cryopreservation vials are transferred to a cryogenic storage unit and stored in the vapor of liquid Nitrogen at a temperature at or below about -135° C. or alternatively vials may be stored in the liquid phase of liquid nitrogen. For example, a suitable cryogenic storage unit includes, but is not limited to, LN2 Freezer MVE 1830 (Chart Industries).
- [00229] Fresh samples of the target stem cell population, or a substantially pure population of target stem cells and thawed samples of the target stem cell population, or a substantially pure population of target stem cells that were previously cryopreserved, may also be analyzed by flow cytometry to analyze cell surface markers, cell viability, and other cell characteristics. A sample of a fresh sample of the target stem cell population, or a substantially pure population of target stem cells may also be analyzed following cell lysis, e.g. using immunoblot analysis and the like.
- [00230] The cryopreserved target stem cell population, or a substantially pure population of target stem cells, may be thawed according to the thawing process described herein. In cases where the target stem cell population, or a substantially pure population of target stem cells, must be thawed, the target stem cell population, or a substantially pure population of target stem cells, may require flow cytometry analysis immediately after thaw or after cells are cultured to assess a certain cell passage. The cryopreserved samples may be agitated in a 37° C. water bath without letting the cells completely thaw. The cells may be transferred into about 1 ml of chilled wash media and mixed by inversion. The sample may be centrifuged at about 2000 rpm for about seven minutes. The supernatant may be removed and the cells resuspended in about 100 µl of wash media (25% HSA, DNase, Heparin and HBSS w/Ca⁺⁺ and Mg⁺⁺).
- [00231] In some embodiments, the resuspended cells may then be centrifuged in a Blood Bank Serofuge for about 1 minute. The supernatant may be decanted and the cells resuspended in about 1.2 ml Sheath fluid and vortexed.
- [00232] Also within the scope of the present invention is moderate to long-term storage of all or part of the target stem cell population, or a substantially pure population of target stem cells, obtained by the methods as disclosed herein, such as moderate or long-term storage in a cell bank. Such moderate to long term storage of cells in a cell bank is disclosed in U.S. Patent Application No. 2003/0054331 and International Patent Publication No. WO 03/024215, which are incorporated by reference in their entireties. At the end of processing, the target stem cell population, or a substantially pure population of target stem cells as disclosed herein, may be loaded into a delivery device, such as a syringe, for placement into the

recipient subject by any means known to one of ordinary skill in the art. In some embodiments, a target stem cell population obtained using the methods as disclosed herein can be suspended in a short term storage medium suitable for infusion into a subject, such as the media disclosed in U.S. Patent No. 5,955,257, which is incorporated herein in its entirety by reference. In some embodiments, the target stem cell populations enriched using the methods as disclosed herein can be stored (e.g., for future therapeutic use by the donor (e.g., allogenic) or by another subject) or directly transplanted, e.g., for use to promote post-surgical healing.

[00233] In one aspect of the invention, the plurality of stem cell populations collected by the methods as disclosed herein can be further sorted into at least two subpopulations which may be cryopreserved separately or together (e.g., in the same vial). In some embodiments, at least two subpopulations of stem cells may be two subpopulations of the stem cells. For example, Fraction 2 can be sorted into at least two subpopulations of cells, for example, but not limited to (1) a stem cell population or a population enriched for VSELs and (2) a non-stem cell population or a population depleted for VSELs. In another example, Fraction 5 can be sorted into at least two subpopulations of cells, for example, but not limited to, (1) a stem cell population of HSCs or a population enriched for HSCs and (2) a stem cell population of MSCs or a population enriched for MSCs, and (3) a non stem cell population or a population depleted for HSCs or MSCs. Furthermore, it is also envisioned that the two subpopulations (i.e., (1) and (2) above) (and optionally population (3)) may be cryopreserved together. In some embodiments, this sorting of stem cells of a fraction into subpopulations can be by positive or negative sorting, or a combination thereof.

[00234] In some embodiments, markers of cell surface proteins can be used to positively select for or negatively select or remove stem cells. Examples of such markers are well known in the art, and are shown in Table 2.

[00235] Table 2:

Table 2	
Cell Type	Markers
Hematopoietic stem cells (HSCs)	C34 ⁺ , CD45 ⁺ , CXCR4 ⁺
Endothelial progenitors	CD34 ⁺ , CD45 ⁺ , CD133 ⁺ , KDR ⁺
Very Small Embryonic-like Cell (VSELs)	CD34 ⁺ , CD133 ⁺ , CXCR4 ⁺ , SSEA4 ⁺
Mesenchymal stem cells (MSCs)	CD34 ⁻ , CD45 ⁻ , CD90 ⁺ , CD105 ⁺ , CD106 ⁺ , CD44 ⁺

[00236] In some embodiments, a target stem cell population, or a substantially pure population of target stem cells obtained by the methods as disclosed herein, can optionally be packaged in a suitable container, in the presence of suitable media. In some embodiments, the package further comprises written instructions for a desired purpose, such as methods of implantation into a subject (and optionally methods of storage and/or methods of thawing if cryopreserved) of the target stem cell population, or a substantially pure population of target stem cells for the improvement or treatment of a disease, or for a treatment protocol for regenerative medicine or therapy.

Cellular therapy:

[00237] In one embodiment of the present invention, the target stem cell populations collected from the peripheral blood of a subject by the methods as disclosed herein can be introduced or transplanted back to the individual when the subject is in need of such cellular therapy.

[00238] A stem cell composition comprising a target stem cell population collected from the peripheral blood of a subject by the methods as disclosed herein can be used to repair, treat, or ameliorate various aesthetic or functional conditions (e.g., defects) through the augmentation of damaged tissues. The target stem cell populations collected from the peripheral blood of a subject by the methods as disclosed herein provide an important resource for rebuilding or augmenting damaged tissues, and thus represent the ability to collect medically useful multiple stem cell populations from a subject from a single source. In a preferred embodiment, the target stem cell populations collected from the peripheral blood of a subject by the methods as disclosed herein can be used in tissue engineering and regenerative medicine for the replacement of body parts that have been damaged by developmental defects, injury, disease, or the wear and tear of aging. The target stem cell populations collected from the peripheral blood of a subject by the methods as disclosed

herein provide a unique system in which the multiple different stem cell populations can be differentiated into different cells and give rise to specific lineages of the same individual or genotypes. The target stem cell populations collected from the peripheral blood of a subject by the methods as disclosed herein therefore provide significant advantages for individualized stem cell therapy.

[00239] In addition, such target stem cell populations collected from the peripheral blood of a subject by the methods as disclosed herein and compositions thereof can be used for augmenting soft tissue not associated with injury by adding bulk to a soft tissue area, opening, depression, or void in the absence of disease or trauma, such as for “smoothing.” Multiple and successive administrations of target stem cell populations collected from the peripheral blood of a subject by the methods as disclosed herein are also embraced by the present invention.

[00240] For stem cell-based treatments, a target stem cell populations collected from the peripheral blood of a subject by the methods as disclosed herein are preferably collected from an autologous or heterologous human or animal source. An autologous animal or human source is more preferred. Stem cell compositions are then prepared and isolated as described herein. To introduce or transplant a target stem cell population collected from the peripheral blood of a subject by the methods as disclosed herein and/or compositions thereof according to the present invention into a human or animal recipient, a suspension of mononucleated cells is prepared. Such suspensions contain concentrations of a target stem cell population collected from the peripheral blood of a subject by the methods as disclosed herein in a physiologically-acceptable carrier, excipient, or diluent. Alternatively, stem cell suspensions may be in serum-free, sterile solutions, such as cryopreservation solutions. Enriched stem cell preparations may also be used. The stem cell suspensions may then be introduced, e.g., via injection, into one or more sites of the donor tissue.

[00241] Concentrated or enriched stem cells may be administered as a pharmaceutically or physiologically acceptable preparation or composition containing a physiologically acceptable carrier, excipient, or diluent, and administered to the tissues of the recipient organism of interest, including humans and non-human animals. The stem cell-containing composition may be prepared by resuspending the cells in a suitable liquid or solution such as sterile physiological saline or other physiologically acceptable injectable aqueous liquids. The amounts of the components to be used in such compositions can be routinely determined by those having skill in the art.

[00242] In some embodiments, the target stem cell populations collected from the peripheral blood of a subject by the methods as disclosed herein or compositions thereof may be administered by placement of the stem cell suspensions onto absorbent or adherent material, e.g., a collagen sponge matrix, and insertion of the stem cell-containing material into or onto the site of interest. Alternatively, the target stem cell populations collected from the peripheral blood of a subject by the methods as disclosed herein may be administered by parenteral routes of injection, including subcutaneous, intravenous, intramuscular and intrasternal. Other modes of administration include, but are not limited to, intranasal, intrathecal, intracutaneous, percutaneous, enteral, and sublingual. In one embodiment, a target stem cell population collected from the peripheral blood of a subject by the methods as disclosed can be administered by endoscopic surgery.

[00243] For injectable administration, a composition comprising a target stem cell population collected from the peripheral blood of a subject by the methods as disclosed herein can be suspended in a sterile solution or suspension or may be resuspended in pharmaceutically- and physiologically-acceptable aqueous or oleaginous vehicles, which may contain preservatives, stabilizers, and material for rendering the solution or suspension isotonic with body fluids (i.e., blood) of the recipient. Non-limiting examples of excipients suitable for use include water, phosphate buffered saline, pH 7.4, 0.15 M aqueous sodium chloride solution, dextrose, glycerol, dilute ethanol, and the like, and mixtures thereof. Illustrative stabilizers are polyethylene glycol, proteins, saccharides, amino acids, inorganic acids, and organic acids, which may be used either on their own or as admixtures. The amounts or quantities, as well as the routes of administration used, are determined on an individual basis, and correspond to the amounts used in similar types of applications or indications known to those of skill in the art.

[00244] Consistent with the present invention, a population of target stem cells collected from the peripheral blood of a subject by the methods as disclosed herein can be administered to body tissues, including epithelial tissue (e.g., skin, lumen, etc.) muscle tissue (e.g., smooth muscle), blood, brain, and various organ tissues such as those organs that are associated with the urological system (e.g., bladder, urethra, ureter, kidneys, etc.).

[00245] According to the general treatment method described herein, a target stem cell population collected from the peripheral blood of a subject by the methods as disclosed herein can comprise other cells, (e.g., a fraction of cells comprising a target stem cell population) and can be administered to a subject, for example, by infusion into the blood stream of a subject through an intravenous (i.v.) catheter, like any other i.v. fluid. Alternatively, however, an

individualized mixture of cells may be generated so as to provide a cellular therapy mixture specific for therapeutic needs of a subject. The comprehensive mixture of cells obtained such as through an apheresis process may be characterized, sorted, and segregated into distinct cell populations. Cell markers such as VSELs, MSC and HSC markers or tissue specific markers may be used to phenotypically characterize the populations of cells collected from the peripheral blood. Using these markers, it is possible to segregate and sort on the basis of cell type. The mixture of cells is thus transformed into populations of cells, which may be broadly classified into two portions: a stem cell portion and a non-stem cell portion. The non-stem cell portion may further be classified into a progenitor cell or fibroblast portion and a functional cell or fully differentiated cell portion. Once the peripheral blood cellular mixture is sorted, the stem cell and non-stem cell portions may be cryopreserved and stored separately. In this manner, a library or repository of distinct cell populations from a subject may be created. Alternatively, stem cell and non-stem cell portions may be cryopreserved together and then sorted and separated prior to use.

[00246] In some embodiments, the target stem cell populations collected from the peripheral blood of a subject by the methods as disclosed herein can be used to generate or differentiate into any population of a cell type that developed from a germ layer (i.e., endoderm, mesoderm, and ectoderm). These include, but are not limited to, differentiated cells, neural progenitor or differentiated cells, glial progenitor or differentiated cells, oligodendrocyte progenitor or differentiated cells, skin progenitor or differentiated cells, hepatic progenitor or differentiated cells, muscle progenitor or differentiated cells, bone progenitor or differentiated cells, mesenchymal stem or progenitor cells, pancreatic progenitor or differentiated cells, progenitor or differentiated chondrocytes, stromal progenitor or differentiated cells, cultured expanded stem or progenitor cells, cultured differentiated stem or progenitor cells, or combinations thereof. Of particular interest are hematopoietic cells, which may include any of the nucleated cells which may be involved with the erythroid, lymphoid or myelomonocytic lineages, as well as myoblasts and fibroblasts. Also of interest are progenitor cells, such as hematopoietic, neural, stromal, muscle (including smooth muscle), hepatic, pulmonary, gastrointestinal, and mesenchymal progenitor cells. Also of interest are differentiated cells, such as, osteoblasts, hepatocytes, granulocytes, chondrocytes, myocytes, adipocytes, neuronal cells, pancreatic, or combinations and mixtures thereof.

[00247] In some embodiments, a target stem cell population collected from the peripheral blood of a subject by the methods as disclosed herein can be combined, recombined, or compounded into a cellular therapy mixture of cells appropriate for treating the disease of a subject and/or

regenerating a specific tissue. A combination of target stem cell populations collected from the peripheral blood of a subject by the methods as disclosed herein, tissue specific progenitor cells, and optionally functional cells can be used, for example, to enhance the engraftment of the transplanted stem cells. Accordingly, in one embodiment, the present invention provides methods and products for using an autologous mixture of one or more target stem cell populations collected from the peripheral blood of a subject by the methods as disclosed herein, either alone, or in combination with other functional cells to enhance engraftment of the transplanted stem cells. In some embodiments, a cellular therapy product may comprise: from about 10% to about 90% of a specific target stem cell population collected from the peripheral blood of a subject by the methods as disclosed herein, about 10% to about 80%, or about 10% to about 60%, or about 10% to about 40%, or about 10% to about 90%, of a target stem cell population collected from the peripheral blood of a subject by the methods as disclosed herein. In some embodiments, a target stem cell population can also comprise a non-stem cell population, such as about from about 5% to about 50% functional cells, about 5% to about 40% functional cells, about 5% to about 30% functional cells, about 5% to about 20% functional cells, or about 5% to about 10% functional cells. Accordingly, in some embodiments, the isolated target stem cell populations provide a library of different stem cell types from a particular individual which can be maintained in culture and/or cryopreserved for future use, e.g., for use alone, or selectively recombined (e.g., custom mixing) for individualized autologous therapeutic applications in regenerative therapy.

[00248] A suitable example of the cellular therapy product described above is the autologous mixture of HSCs and MSCs or other custom mixing of autologous stem cell populations, or with other functional cells of the hematopoietic system. Another example is a cellular therapy product comprising an autologous mixture of PBSCs, myocardial progenitor cells, and optionally myocardial cells.

[00249] Accordingly, in another embodiment, there is provided a method of treating a patient in need thereof comprising administering to a subject an autologous mixture of one or more target stem cell populations, either alone or in combination (either separately or in an admixture) collected from the peripheral blood of a subject by the methods as disclosed herein.

[00250] In another aspect of the present invention, a target stem cell populations collected from the peripheral blood of a subject by the methods as disclosed herein can be stored in a cell bank to support an elective healthcare insurance model to effectively protect members of the population from future diseases. An individual subject can elect to have his or her own stem cell populations collected from the peripheral blood, processed and preserved, while he or she is in healthy state, for future distribution for his or her healthcare needs.

[00251] Accordingly, in one embodiment, target stem cell populations collected from the peripheral blood of a subject by the methods as disclosed herein are “banked” for future use, at a stem cell bank or depository or storage facility, or any place where the target stem cell populations are kept for safekeeping. The storage facility may be designed in such a way that the target stem cell populations collected from the peripheral blood of a subject by the methods as disclosed herein are kept safe in the event of a catastrophic event such as a nuclear attack. In some embodiments, the storage facility might be underground, in caves or in silos. In other embodiments, it may be on the side of a mountain or in outer space. The storage facility may be encased in a shielding material such as lead.

[00252] According to another embodiment, there is provided a process of stem cell banking with four steps. Step A involves administering one or more mobilizing agents to a subject to increase the amount of stem cell populations in the peripheral blood of the subject. Step B involves collecting at least one population of target stem cell populations from the peripheral blood by the methods as disclosed herein using an elutriation process, wherein said subject has no immediate perceived health condition, e.g., no condition where the subject is requiring treatment using his autologous transplantation of their own target stem cell populations. Step C involves preserving the at least one population of a target stem cell population collected from the peripheral blood by the methods as disclosed herein as a preserved populations of cells. Step D involves retrieving the preserved populations of target stem cells collected by the methods as disclosed herein for autologous transplantation of the target stem cells into the subject.

Use of target stem cell populations for personalized medicine diagnostic and prognostic assays.

[00253] Another aspect of the present invention relates to the use of the target stem cell populations obtained without using positive selection methods from a subjects peripheral blood by the methods as disclosed herein, for personalized medicine applications such as autologous therapeutic use, as well as in a personalized assay to assess the effect of a

person's diet, pharmacogenetics, neurochemicals, and/or lifestyle on the function and viability of stem cell populations. Such assays are currently well known in the art, and include high throughput screens where a compound, agent, or environmental stimulus is contacted with a stem cell and the effect on the function or viability of the compound, agent or environmental stimulus on the function (e.g., differentiation potential, propagation, survival) and viability can be measured, and the result compared to a reference control sample (e.g., in the absence of a compound, or a positive control sample, such as the presence of BMP6 or other growth factor and the like).

[00254] In one embodiment of the invention, a target stem cell population obtained without using positive selection methods from a subject's peripheral blood by the methods as disclosed herein can be used as an individualized assay for the study and understanding of signaling pathways of the subject's own stem cell populations' growth and differentiation. The use of the subject's target stem cells obtained by the methods as disclosed herein are useful to aid the development of therapeutic applications for congenital and adult heart failure. The use of such subject's target stem cells obtained by the methods as disclosed herein enables the study of specific differentiation into different lineages, e.g., osteogenic lineages or cardiac lineages, without the need and complexity of time consuming animal models. In another embodiment, the subject's target stem cells obtained by the methods as disclosed herein can be genetically modified to carry specific disease and/or pathological traits and phenotypes of a particular disease or disorder.

[00255] In one embodiment, the assay comprises a plurality of the subject's different target stem cell populations obtained by the methods as disclosed herein, or their differentiated progeny. In one embodiment, the assay can be used for the study of differentiation pathways of a subject's different target stem cell populations, for example but not limited to the differentiation along the cardiomyocyte lineage, smooth muscle lineage, endothelial lineage, and subpopulations of these lineages.

[00256] In another embodiment, the assay can be used to study a pathological characteristic of the subject's different target stem cell populations, for example, a disease and/or genetic characteristic associated with a disease or disorder. In some embodiments, the disease or disorder is a wound healing disorder, bone disorder or a cardiovascular disorder or disease. In some embodiments, the subject's different target stem cell populations have been genetically engineered to comprise the characteristics associated with a disease or disorder. Such methods to genetically engineer stem cells are well known by those in the art, and include

introducing nucleic acids into the cell by means of transfection by, for example, but not limited to, use of viral vectors or by other means known in the art.

[00257] In one embodiment, one can use these target stem cells to more closely tailor the individuals treatment. For example, by administering various drug protocols being administered or proposed for administration, one can directly determine potential drug interaction problems based upon that individual's unique genetic makeup and environmental factors the individual encounters. In this way, protocols, dosages, or timing of administrations can be changed to obtain the best effect.

[00258] In some embodiments, as the target stem cells are specific to the subject's genetic make-up, the use of the MSCs provide a tool for pharmacogenetic analysis of the target stem cell populations. For example, a subject's target stem cells may harbor a particular variant which results in a distinct pathological characteristic. For example, as the stem cells are from the subject (e.g., autologous), they may have an undesirable pathological characteristic, e.g., mutation and/or polymorphism, which contributes to disease pathology, or results in a poor or enhanced response to a therapeutic drug. Therefore, the target stem cells obtained by the methods disclosed herein can be used to assess the response of the subject's stem cells to particular compounds, agents, and in some cases be useful for determining treatment regimens, (e.g., effective treatment drugs, doses and durations) and in some cases, prognosis for disease. In such an embodiment, the methods of the invention can be used to screen the target stem cells for agents which alleviate the pathology, or agents which positively affect the propagation or function of the target stem cells.

[00259] In alternative embodiments, the methods of the invention can be used to screen for agents which cause a different response on a population of the subject's target stem cells (due to an inherent genetic make up or a particular mutation and/or polymorphism) as compared with stem cells from other subjects (e.g., without the mutation and/or polymorphism), therefore the methods can be used for example, to assess an effect of a particular drug and/or agent on subject's stem cell population as compared to other people and/or stem cell populations, therefore acting as a high-throughput screen for personalized medicine and/or pharmacogenetics. The manner in which the target stem cells respond to an agent, particularly a pharmacologic agent, including the timing of responses, is an important reflection of the physiologic state of the cell.

[00260] In some embodiments, the subject's different target stem cell populations can be easily manipulated in experimental systems that offer the advantages of targeted lineage differentiation as well as clonal homogeneity and the ability to manipulate external

environments. Furthermore, due to the ethical unacceptability of experimentally altering a human germ line, the ES cell transgenic route is not available for experiments that involve the manipulation of human genes. Gene targeting in a human subject's different target stem cell populations allows important applications in areas where the system cannot be tested *in vivo* due to ethical issues, and can adequately recapitulate human biology or disease processes for that particular subject or individual.

[00261] In another embodiment, the subject's different target stem cell populations can be used to prepare a cDNA library relatively uncontaminated with cDNA that is preferentially expressed in cells from other lineages.

[00262] The subject's different target stem cell populations can also be used to prepare antibodies that are specific for markers of cardiomyocytes and their precursors. Polyclonal antibodies can be prepared by injecting a vertebrate animal with cells of this invention in an immunogenic form. Production of monoclonal antibodies is described in such standard references as U.S. Pat. Nos. 4,491,632; 4,472,500 and 4,444,887, and Methods in Enzymology 73B:3 (1981). Specific antibody molecules can also be produced by contacting a library of immunocompetent cells or viral particles with the target antigen, and growing out positively selected clones. See Marks et al., New Eng. J. Med. 335:730, 1996, and McGuinness et al., Nature Biotechnol. 14:1449, 1996. A further alternative is reassembly of random DNA fragments into antibody encoding regions, as described in EP patent application 1,094,108 A.

[00263] The antibodies in turn can be used to identify or rescue (for example, restore the phenotype) cells of a desired phenotype from a mixed cell population, for purposes such as costaining during immunodiagnosis using tissue samples, and isolating precursor cells from a terminally differentiated subject's different target stem cell populations and cells of other lineages.

[00264] In another embodiments, the subject's different target stem cell populations can be used for the examination of the gene expression profile during and following differentiation of the subject's different target stem cell populations. The expressed set of genes may be compared against other subsets of stem cell populations from the same or a different subject or control stem cell line sample as known in the art. Any suitable qualitative or quantitative methods known in the art for detecting specific mRNAs can be used. mRNA can be detected by, for example, hybridization to a microarray, *in situ* hybridization in tissue sections, by reverse transcriptase-PCR, or in Northern blots containing poly A+ mRNA. One of skill in

the art can readily use these methods to determine differences in the molecular size or amount of mRNA transcripts between two samples.

[00265] Any suitable method for detecting and comparing mRNA expression levels in a sample can be used in connection with the methods of the invention. For example, mRNA expression levels in a sample can be determined by generation of a library of expressed sequence tags (ESTs) from a sample. Enumeration of the relative representation of ESTs within the library can be used to approximate the relative representation of a gene transcript within the starting sample. The results of EST analysis of a test sample can then be compared to EST analysis of a reference sample to determine the relative expression levels of a selected polynucleotide, particularly a polynucleotide corresponding to one or more of the differentially expressed genes described herein.

[00266] Alternatively, gene expression in a test sample can be performed using serial analysis of gene expression (SAGE) methodology (Velculescu et al., Science (1995) 270:484). In short, SAGE involves the isolation of short unique sequence tags from a specific location within each transcript. The sequence tags are concatenated, cloned, and sequenced. The frequency of particular transcripts within the starting sample is reflected by the number of times the associated sequence tag is encountered with the sequence population.

[00267] Gene expression in a test sample can also be analyzed using differential display (DD) methodology. In DD, fragments defined by specific sequence delimiters (e.g., restriction enzyme sites) are used as unique identifiers of genes, coupled with information about fragment length or fragment location within the expressed gene. The relative representation of an expressed gene within a sample can then be estimated based on the relative representation of the fragment associated with that gene within the pool of all possible fragments. Methods and compositions for carrying out DD are well known in the art, see, e.g., U.S. Patent No. 5,776,683; and U.S. Patent No. 5,807,680. Alternatively, gene expression can be estimated in a sample using hybridization analysis, which is based on the specificity of nucleotide interactions. Oligonucleotides or cDNA can be used to selectively identify or capture DNA or RNA of specific sequence composition, and the amount of RNA or cDNA hybridized to a known capture sequence determined qualitatively or quantitatively, to provide information about the relative representation of a particular message within the pool of cellular messages in a sample. Hybridization analysis can be designed to allow for concurrent screening of the relative expression of hundreds to thousands of genes by using, for example, array-based technologies having high density formats, including filters, microscope slides, or microchips, or solution-based technologies that use spectroscopic

analysis (e.g., mass spectrometry). One exemplary use of arrays in the diagnostic methods of the invention is described below in more detail.

[00268] Hybridization to arrays may be performed, where the arrays can be produced according to any suitable methods known in the art. For example, methods of producing large arrays of oligonucleotides are described in U.S. Patent No. 5,134,854, and U.S. Patent No. 5,445,934 using light-directed synthesis techniques. Using a computer-controlled system, a heterogeneous array of monomers is converted, through simultaneous coupling at a number of reaction sites, into a heterogeneous array of polymers. Alternatively, microarrays are generated by deposition of pre-synthesized oligonucleotides onto a solid substrate, for example, as described in PCT published application no. WO 95/35505. Methods for collection of data from hybridization of samples with an array are also well known in the art. For example, the polynucleotides of the cell samples can be generated using a detectable fluorescent label, and hybridization of the polynucleotides in the samples detected by scanning the microarrays for the presence of the detectable label. Methods and devices for detecting fluorescently marked targets on devices are known in the art. Generally, such detection devices include a microscope and light source for directing light at a substrate. A photon counter detects fluorescence from the substrate, while an x-y translation stage varies the location of the substrate. A confocal detection device that can be used in the subject methods is described in U.S. Patent No. 5,631,734. A scanning laser microscope is described in Shalon et al., *Genome Res.* (1996) 6:639. A scan, using the appropriate excitation line, is performed for each fluorophore used. The digital images generated from the scan are then combined for subsequent analysis. For any particular array element, the ratio of the fluorescent signal from one sample is compared to the fluorescent signal from another sample, and the relative signal intensity determined. Methods for analyzing the data collected from hybridization to arrays are well known in the art. For example, where detection of hybridization involves a fluorescent label, data analysis can include the steps of determining fluorescent intensity as a function of substrate position from the data collected, removing outliers, i.e., data deviating from a predetermined statistical distribution, and calculating the relative binding affinity of the targets from the remaining data. The resulting data can be displayed as an image with the intensity in each region varying according to the binding affinity between targets and probes. Pattern matching can be performed manually or can be performed using a computer program. Methods for preparation of substrate matrices (e.g., arrays), design of oligonucleotides for use with such matrices, labeling of probes,

hybridization conditions, scanning of hybridized matrices, and analysis of patterns generated, including comparison analysis, are described in, for example, U.S. Patent No. 5,800,992.

[00269] In the use of the target stem cells obtained by the methods as disclosed herein, a stem cell population or a combination of stem cell populations are contacted with the agent of interest, and the effect of the agent assessed by monitoring output parameters, such as expression of markers, cell viability, differentiation characteristics, multipotent capacity and the like. The target stem cells may be freshly isolated using the methods as disclosed herein, or in some embodiments, cultured, cryopreserved, or genetically engineered prior to using in an assay. The target stem cells can be environmentally induced variants of clonal cultures: e.g., split into independent cultures and grown under distinct conditions, for example, with or without virus; in the presence or absence of other cytokines or combinations thereof. Alternatively, the target stem cells may be variants with a desired pathological characteristic. For example, as the target stem cells are from the subject (i.e., autologous) they have a desired pathological characteristic, e.g., mutation and/or polymorphism which contribute to disease pathology. Therefore, the pool of target stem cells obtained by the methods as disclosed herein can be used to assess the subject's target stem cells' response to particular compounds, agents, and in some cases may be useful for a prognosis for disease. In such an embodiment, the methods of the invention can be used to screen the target stem cell populations for agents which alleviate the pathology.

[00270] In alternative embodiments, the methods of the invention can be used to screen for agents where some target stem cell populations comprising a particular mutation and/or polymorphism respond differently as compared with the same type of stem cells without the mutation and/or polymorphism. Therefore, the methods can be used, for example, to assess an effect of a particular drug and/or agent on a subject's target stem cell population as compared to other people and/or cells, therefore acting as a high-throughput screen for personalized medicine and/or pharmacogenetics. The manner in which cells respond to an agent, particularly a pharmacologic agent, including the timing of responses, is an important reflection of the physiologic state of the cell.

[00271] The agent used in the screening method can be selected from a group of a chemical, small molecule, chemical entity, nucleic acid sequences, an action, nucleic acid analogues or protein or polypeptide or analogue of fragment thereof. In some embodiments, the nucleic acid is DNA or RNA, and nucleic acid analogues, for example, can be PNA, pcPNA and LNA. A nucleic acid may be single or double stranded, and can be selected from a group comprising; nucleic acid encoding a protein of interest, oligonucleotides, PNA, etc. Such

nucleic acid sequences include, for example, but are not limited to, nucleic acid sequence encoding proteins that act as transcriptional repressors, antisense molecules, ribozymes, small inhibitory nucleic acid sequences, for example, but not limited to RNAi, shRNAi, siRNA, micro RNAi (mRNAi), antisense oligonucleotides, etc.. A protein and/or peptide agent or fragment thereof, can be any protein of interest, for example, but not limited to, mutated proteins, therapeutic proteins, or truncated proteins, wherein the protein is normally absent or expressed at lower levels in the cell. Proteins of interest can be selected from a group comprising: mutated proteins, genetically engineered proteins, peptides, synthetic peptides, recombinant proteins, chimeric proteins, antibodies, humanized proteins, humanized antibodies, chimeric antibodies, modified proteins and fragments thereof. The agent may be applied to the media, where it contacts the cell (such as stem cells and/or mesenchymal cells) and induces its effects. Alternatively, the agent may be intracellular, i.e., within the cell (such as stem cell and/or mesenchymal cells), as a result of introduction of the nucleic acid sequence into the cell and its transcription, resulting in the production of the nucleic acid and/or protein agent within the cell. An agent also encompasses any action and/or event the cells are subjected to. As a non-limiting examples, an action can comprise any action that triggers a physiological change in the cell, for example, but not limited to, heat-shock, ionizing irradiation, cold-shock, electrical impulse, light and/or wavelength exposure, UV exposure, pressure, stretching action, increased and/or decreased oxygen exposure, exposure to reactive oxygen species (ROS), ischemic conditions, fluorescence exposure etc. Environmental stimuli also include intrinsic environmental stimuli defined below. The exposure to agent may be continuous or non-continuous.

[00272] The term “agent” refers to any chemical, entity or moiety, including without limitation synthetic and naturally-occurring non-proteinaceous entities. In certain embodiments, the compound of interest is a small molecule having a chemical moiety. For example, chemical moieties included unsubstituted or substituted alkyl, aromatic, or heterocyclyl moieties including macrolides, leptomycins and related natural products or analogues thereof. Compounds can be known to have a desired activity and/or property, or can be selected from a library of diverse compounds.

[00273] In some embodiments, the agent is an agent of interest including known and unknown compounds that encompass numerous chemical classes, primarily organic molecules, which may include organometallic molecules, inorganic molecules, genetic sequences, etc.. An important aspect of the invention is to evaluate candidate drugs, including toxicity testing and the like. Candidate agents also include organic molecules comprising functional groups

necessary for structural interactions, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, frequently at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules, including peptides, polynucleotides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

[00274] Also included as agents are pharmacologically active drugs, genetically active molecules, etc.. Compounds of interest include, for example, chemotherapeutic agents, hormones or hormone antagonists, growth factors or recombinant growth factors and fragments and variants thereof. Exemplary of pharmaceutical agents suitable for this invention are those described in, "The Pharmacological Basis of Therapeutics," Goodman and Gilman, McGraw-Hill, New York, N.Y., (1996), Ninth edition, under the sections: Water, Salts and Ions; Drugs Affecting Renal Function and Electrolyte Metabolism; Drugs Affecting Gastrointestinal Function; Chemotherapy of Microbial Diseases; Chemotherapy of Neoplastic Diseases; Drugs Acting on Blood-Forming organs; Hormones and Hormone Antagonists; Vitamins, Dermatology; and Toxicology, all incorporated herein by reference. Also included are toxins, and biological and chemical warfare agents, for example, see Somani, S. M. (Ed.), "Chemical Warfare Agents," Academic Press, New York, 1992).

[00275] The agents include all of the classes of molecules described above, and may further comprise samples of unknown content. Of interest are complex mixtures of naturally occurring compounds derived from natural sources such as plants. While many samples will comprise compounds in solution, solid samples that can be dissolved in a suitable solvent may also be assayed. Samples of interest include environmental samples, e.g., ground water, sea water, mining waste, etc.; biological samples, e.g., lysates prepared from crops, tissue samples, etc.; manufacturing samples, e.g., time course during preparation of pharmaceuticals; as well as libraries of compounds prepared for analysis; and the like. Samples of interest include compounds being assessed for potential therapeutic value, i.e., drug candidates.

[00276] Parameters are quantifiable components of target stem cells, particularly components that can be accurately measured, desirably in a high throughput system. A parameter can be any cell component or cell product including cell surface determinant, receptor, protein or conformational or posttranslational modification thereof, lipid, carbohydrate, organic or inorganic molecule, nucleic acid, e.g., mRNA, DNA, etc. or a portion derived from such a

cell component or combinations thereof. While most parameters will provide a quantitative readout, in some instances a semi-quantitative or qualitative result will be acceptable.

Readouts may include a single determined value, or may include mean, median value or the variance, etc.. Characteristically a range of parameter readout values will be obtained for each parameter from a multiplicity of the same assays. Variability is expected and a range of values for each of the set of test parameters will be obtained using standard statistical methods with a common statistical method used to provide single values.

[00277] Compounds, including candidate agents, are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds, including biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc., to produce structural analogs.

[00278] Agents are screened for effect on the stem cell population by adding the agent to at least one and usually a plurality of stem cell samples, usually in conjunction with cells lacking the agent. The change in parameters in response to the agent is measured, and the result evaluated by comparison to reference cultures, e.g., in the presence and absence of the agent, obtained with other agents, etc..

[00279] In some embodiments, the agents are conveniently added in solution, or readily soluble form, to the medium of stem cells in culture. The agents may be added in a flow-through system, as a stream, intermittent or continuous, or alternatively, by adding a bolus of the compound, singly or incrementally, to an otherwise static solution. In a flow-through system, two fluids are used, where one is a physiologically neutral solution, and the other is the same solution with the test compound added. The first fluid is passed over the stem cells, followed by the second. In a single solution method, a bolus of the test compound is added to the volume of medium surrounding the cells. The overall concentrations of the components of the culture medium should not change significantly with the addition of the bolus, or between the two solutions in a flow through method. In some embodiments, agent formulations do not include additional components, such as preservatives, that may have a significant effect on

the overall formulation. Thus preferred formulations consist essentially of a biologically active compound and a physiologically acceptable carrier, e.g., water, ethanol, DMSO, etc.. However, if a compound is liquid without a solvent, the formulation may consist essentially of the compound itself.

[00280] A plurality of assays may be run in parallel with different agent concentrations to obtain a differential response to the various concentrations. As known in the art, determining the effective concentration of an agent typically uses a range of concentrations resulting from 1:10, or other log scale, dilutions. The concentrations may be further refined with a second series of dilutions, if necessary. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration or below the level of detection of the agent or at or below the concentration of agent that does not give a detectable change in the phenotype.

[00281] Optionally, the stem cell population used in the screen can be manipulated to express desired gene products. Gene therapy can be used to either modify a cell to replace a gene product or add or knockdown a gene product. In some embodiments, the genetic engineering is done to facilitate regeneration of tissue, to treat disease, or to improve survival of the cells following implantation into a subject (e.g., prevent rejection). Alternatively, in one embodiment the mesenchymal cells are genetically engineered and transfected prior to their use as a feeder layer for the stem cells, or alternatively, the mesenchymal cells can be transfected while they function as feeder layer for stem cells. Techniques for transfecting cells are known in the art.

[00282] A skilled artisan could envision a multitude of genes which would convey beneficial properties to the transfected mesenchymal cells or, more indirectly, to the recipient stem cells and/or subject if the stem cells are used in transplantation (discussed in more detail below). The added gene may ultimately remain in the recipient cell and all its progeny, or may only remain transiently, depending on the embodiment. For example, genes encoding angiogenic factors could be transfected into progenitor cells isolated from smooth muscle. Such genes would be useful for inducing collateral blood vessel formation as the smooth muscle tissue is regenerated. In some situations, it may be desirable to transfect the cell with more than one gene.

[00283] In some instances, it is desirable to have the gene product secreted. In such cases, the gene product preferably contains a secretory signal sequence that facilitates secretion of the protein. For example, if the desired gene product is an angiogenic protein, a skilled artisan could either select an angiogenic protein with a native signal sequence, e.g., VEGF, or can modify the gene product to contain such a sequence using routine genetic manipulation (see,

e.g., Nabel et al., 1993).

[00284] The desired gene can be transfected into the cell using a variety of techniques.

Preferably, the gene is transfected into the cell using an expression vector. Suitable expression vectors include plasmid vectors (such as those available from Stratagene, Madison Wis.), viral vectors (such as replication defective retroviral vectors, herpes virus, adenovirus, adenovirus associated virus, and lentivirus), and non-viral vectors (such as liposomes or receptor ligands).

EXAMPLES

[00285] Reference will be made in detail to the embodiments of the invention, examples of which are illustrated in some of the accompanying drawings. In some embodiments, the present invention includes an ELUTRA® centrifuge manufactured by Gambro BCT, Inc. of Lakewood, Colorado. Although embodiments of this invention are described in combination with the ELUTRA® centrifuge, this reference is made for exemplary purposes only and is not intended to limit the invention in any sense. It is understood that other centrifuges could be used with embodiments of the invention, including but not limited to, the COBE® Spectra apheresis system, the TRIMA® system and the TRIMA ACCEL® system, also manufactured by Gambro BTC Inc., as well as other elutriation devices used to separate blood components.

[00286] Example 1

[00287] Apheresis mobilized PB cells were subjected to elutriation to separate/enrich MSCs. A group of 28 donors were studied after mobilization for two consecutive days of G-CSF injection (480µg/day subcutaneously) and collection of total nucleated cells (TNC) by apheresis for 3-4 hours on the third day.

[00288] **Size-based MSC enrichment by Elutriation:** Fresh apheresed cells were lysed with 1X BD Pharm Lyse Buffer, in a ratio of approximately 1:10 (vol/vol) to remove red blood cells. After washing, cells were counted, and $2-2.5 \times 10^{10}$ total nucleated cells were loaded onto the ELUTRA® Cell Separation System (CaridianBCT) at a concentration of 1×10^8 cells/ml. Cells were then collected in 900 ml PBS + 0.5% HSA media in each bag at different flow rates. Typically, six fractions were collected with a centrifugation speed of 2400 rpm. Finally, cells from all fractions were transferred into tubes and spun down at 600 x g for 15 minutes. Size characteristics of the fractions were confirmed by evaluating SSC and FSC (Figure 3).

[00289] **CFU-F Assay:** ELUTRA® fractions were tested for a possible enrichment in MSCs by plating cells in a CFU-F assay. 2 million cells per each fraction were plated onto a 100-mm tissue culture dish and supplemented with 10 mL of DMEM + 10% FBS media. Cultures were left undisturbed in a 37° C incubator with 5% CO₂ for two weeks. After two weeks, cultures were fixed with methanol and stained with Giemsa. Adherent colonies characteristic of MSCs developed from fractions 4 and 5 (Figure 4).

[00290] **Staining of fractionated apheresis populations:** Flow cytometry analysis was used to determine whether certain ELUTRA fractions were enriched in MSCs. Samples from the ELUTRA® fractions were stained using two panels of antibodies: panel 1: CD45, CD105, CD90, and panel 2: CD271. Staining was performed in PBS containing 0.5% Human Serum Albumin for 30 minutes on ice. Cells were washed and analyzed by FACS as CD45⁺/CD105⁺, CD45⁺/CD90⁺ and CD45⁺/CD271⁺. Fractions 4 and 5 were enriched for cells characterized by CD45⁺/CD105⁺, CD45⁺/CD90⁺, and CD271⁺ as compared to the apheresis product starting population (Figure 5).

[00291] **G-CSF mobilized MSCs into peripheral blood.** Figure 6 shows that the proportion of MSCs increased post mobilization and in the resulting apheresis product. Here, MSCs were identified by detecting cells expressing CD45⁺/CD31⁺/CD105⁺/CD73⁺ or CD45⁺/CD34⁺/CD90⁺/CD105⁺/CD29⁺.

REFERENCES

[00292] All references, patents and patent applications cited herein and throughout the specification are herewith incorporated by reference in their entirety.

CLAIMS

1. A method for separating a target cell population from peripheral blood comprising:

(a) flowing a peripheral blood sample from a subject through a fluid chamber of an elutriation apparatus at a first flow rate, wherein the first flow rate is selected so that cells in the peripheral blood sample smaller than the target cell population flow through the fluid chamber and the target cell population is retained in the fluid chamber;

(b) increasing the first flow rate to a second flow rate so that the target cell population flows through the fluid chamber; and

(c) collecting the target cell population as it flows through the fluid chamber;

where steps (a), (b), and (c) do not comprise the use of positive selection techniques.

2. The method of claim 1 where (i) the first flow rate is selected so that cells in the peripheral blood sample smaller than Very Small Embryonic Like stem cells (VSELs) flow through the fluid chamber and VSELs are retained in the fluid chamber and (ii) the second flow rate is selected so that VSELs flow through the fluid chamber.

3. The method of claim 2 where (i) the first flow rate is about 35 ml/min and the second flow rate is about 50-70 ml/min.

4. The method of claim 1 where (i) the first flow rate is selected so that cells in the peripheral blood sample smaller than mesenchymal stem cells (MSCs) flow through the fluid chamber and MSCs are retained in the fluid chamber and (ii) the second flow rate is selected so that MSCs flow through the fluid chamber.

5. The method of claim 4 where (i) the first flow rate is about 100 ml/min and the second flow rate is about 110-120 ml/min.

6. The method of claim 1 where (i) the first flow rate is selected so that cells in the peripheral blood sample smaller than hematopoietic stem cells (HSCs) flow through the fluid chamber and HSCs are retained in the fluid chamber and (ii) the second flow rate is selected so that HSCs flow through the fluid chamber.

7. The method of claim 6 where (i) the first flow rate is about 90 ml/min and the second flow rate is about 100 ml/min.
8. The method of any one of claims 1-7 where the positive selection technique is immunoselection or immunodensity selection.
9. The method of any one of claims 1-8 where the peripheral blood sample is a human peripheral blood sample.
10. The method of any one of claims 1-9 where the peripheral blood sample is a mobilized peripheral blood sample.
11. The method of claim 10 where the peripheral blood sample is obtained from a subject who has been administered a mobilizing agent for 4 days or less.
12. The method of any one of claims 1-11 where the collected target cell population is cryopreserved.
13. The method of any one of claims 1-12 where the collected target cell populations is administered to a subject.
14. The method of claim 13 where the collected target cell population is administered to a subject from whom the peripheral blood was originally obtained.
15. The method of claim 13 or 14 where the collected target cell populations has been expanded *in vitro* prior to administering to the subject.
16. The method of claim 15 where the subject is a human subject.
17. The method of any one of claims 13-15 where the collected target cell population is VSELs.

18. The method of any one of claims 13-15 where the collected target cell population is MSCs.

19. The method of any one of claims 13-15 where the collected target cell population is HSCs.

20. A method for separating target cell populations from peripheral blood comprising:

(a) flowing a peripheral blood sample from a subject through a fluid chamber of an elutriation apparatus at a first flow rate, wherein the first flow rate is selected so that a first target cell population flows through the fluid chamber and is collected while a first remainder of the peripheral blood sample is retained in the fluid chamber; and

(b) increasing the first flow rate to a second flow rate so that a second target cell population flows through the fluid chamber and is collected while a second remainder of the peripheral blood sample is retained in the fluid chamber;

where steps (a) and (b) do not comprise the use of positive selection techniques.

21. The method of claim 20 further comprising:

(c) increasing the second flow rate to a third flow rate so that a third target cell population flows through the fluid chamber and is collected while a third remainder of the peripheral blood sample is retained in the fluid chamber;

where step (c) does not comprise the use of positive selection techniques.

22. The method of claim 21 further comprising:

(d) increasing the third flow rate to a fourth flow rate so that a fourth target cell population flows through the fluid chamber and is collected while a fourth remainder of the peripheral blood sample is retained in the fluid chamber;

where step (d) does not comprise the use of positive selection techniques.

23. The method of claim 22 further comprising:

(e) increasing the fourth flow rate to a fifth flow rate so that a fifth target cell population flows through the fluid chamber and is collected while a fifth remainder of the peripheral blood sample is retained in the fluid chamber;

where step (e) does not comprise the use of positive selection techniques.

24. The method of claim 23 further comprising:

(f) increasing the fifth flow rate to a sixth flow rate so that a sixth target cell population flows through the fluid chamber and is collected while a sixth remainder of the peripheral blood sample is retained in the fluid chamber;
where step (f) does not comprise the use of positive selection techniques.

25. The method of claim 24 further comprising:

(g) increasing the sixth flow rate to a seventh flow rate so that a seventh target cell population flows through the fluid chamber and is collected while a seventh remainder of the peripheral blood sample is retained in the fluid chamber;
where step (g) does not comprise the use of positive selection techniques.

26. The method of claim 20 where (i) the first flow rate is selected so that the first target cell population is cells smaller than Very Small Embryonic Like stem cells (VSELs) and (ii) the second flow rate is selected so that the second target cell population is VSELs.

27. The method of claim 26 where (i) the first flow rate is about 35 ml/min and the second flow rate is about 50-70 ml/min.

28. The method of claim 22 where (i) the third flow rate is selected so that the first target cell population is cells smaller than hematopoietic stem cells (HSCs) and (ii) the fourth flow rate is selected so that the second target cell population is HSCs.

29. The method of claim 28 where (i) the third flow rate is about 90 ml/min and the fourth flow rate is about 100 ml/min.

30. The method of claim 23 where (i) the fourth flow rate is selected so that the first target cell population is cells smaller than mesenchymal stem cells (MSCs) and (ii) the fifth flow rate is selected so that the second target cell population is MSCs.

31. The method of claim 30 where (i) the third flow rate is about 100 ml/min and the fourth flow rate is about 110-120 ml/min.

32. The method of claim 25 where:

- (i) the first flow rate is about 35 ml/min and the first target cell population is platelets;
- (ii) the second flow rate is about 50 ml/min and the second target cell population is VSELs;
- (iii) the third flow rate is about 70 ml/min and the third target cell population is VSELs;
- (iv) the fourth flow rate is about 90 ml/min and the fourth target cell population is red blood cells;
- (v) the fifth flow rate is about 100 ml/min and the fifth target cell population is HSCs;
- (vi) the sixth flow rate is about 110 ml/min and the sixth target cell population is MSCs; and
- (vii) the seventh flow rate is about 120 ml/min and the seventh target cell population is MSCs.

33. The method of any one of claims 20-32 where the positive selection technique is immunoselection or immunodensity selection.

34. The method of any one of claims 20-33 where the peripheral blood sample is a human peripheral blood sample.

35. The method of any one of claims 20-34 where the peripheral blood sample is a mobilized peripheral blood sample.

36. The method of claim 35 where the peripheral blood sample is obtained from a subject who has been administered a mobilizing agent for 4 days or less.

37. The method of any one of claims 20-36 where the collected target cell population is cryopreserved.

38. The method of any one of claims 20-37 where at least one of the collected target cell populations is administered to a subject.

39. The method of claim 38 where wherein at least one of the collected target cell populations are administered to a subject from whom the peripheral blood was originally obtained.

40. The method of claim 38 or 39 where the at least one of the collected target cell populations has been expanded *in vitro* prior to administering to the subject.

41. The method of any one of claims 38-40 where the subject is a human subject.
42. The method of any one of claims 38-41 where the at least one of the collected target cell populations is VSELs.
43. The method of any one of claims 38-41 where the at least one of the collected target cell populations is MSCs.
44. The method of any one of claims 38-41 where the at least one of the collected target cell populations is HSCs.
45. A method for separating target cell populations from peripheral blood comprising:
- (a) flowing a peripheral blood sample from a subject through a fluid chamber of an elutriation apparatus at a first flow rate, wherein the first flow rate is selected so that a first target cell population flows through the fluid chamber and is collected while a first remainder of the peripheral blood sample is retained in the fluid chamber;
 - (b) increasing the first flow rate to a second flow rate so that a second target cell population flows through the fluid chamber and is collected while a second remainder of the peripheral blood sample is retained in the fluid chamber; and
 - (c) optionally, repeating step (b) until a desired number of target cell populations are collected;
- where steps (a), (b), and (c) do not comprise the use of positive selection techniques.
46. The method of any one of claims 2, 3, 17, 26, 27, or 42, where the peripheral blood sample is a human peripheral blood sample with a hematocrit range of about 2-3%.
47. A method of reducing granulocytes in a composition of peripheral blood VSELs from a subject, comprising separating VSELs from other somatic cells by an apheresis procedure, wherein the hematocrit of the separated cells is 2-3%.
48. A method of increasing the yield of VSELs obtained in the downstream processing of an apheresis product by selecting the hematocrit range to be 2-3%, thereby reducing the presence of granulocytes in the collected apheresis product.

49. VSELs product by the method of any one of claims 2, 3, 26, or 27.

50. MSCs product by the method of any one of claims 4, 5, 30, or 31.

51. HSCs product by the method of any one of claims 6, 7, 28, or 29.

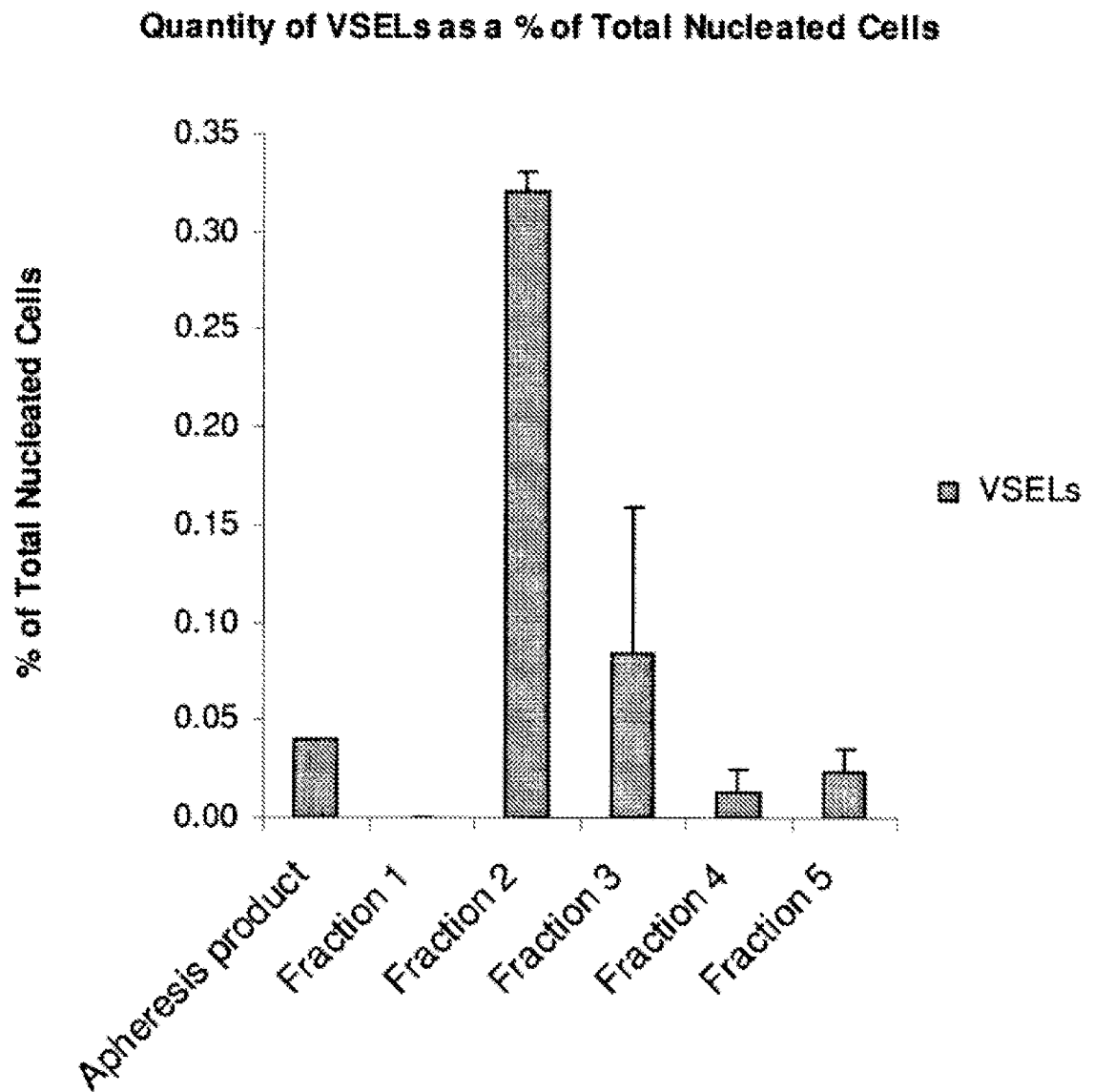
FIGURE 1

FIGURE 2

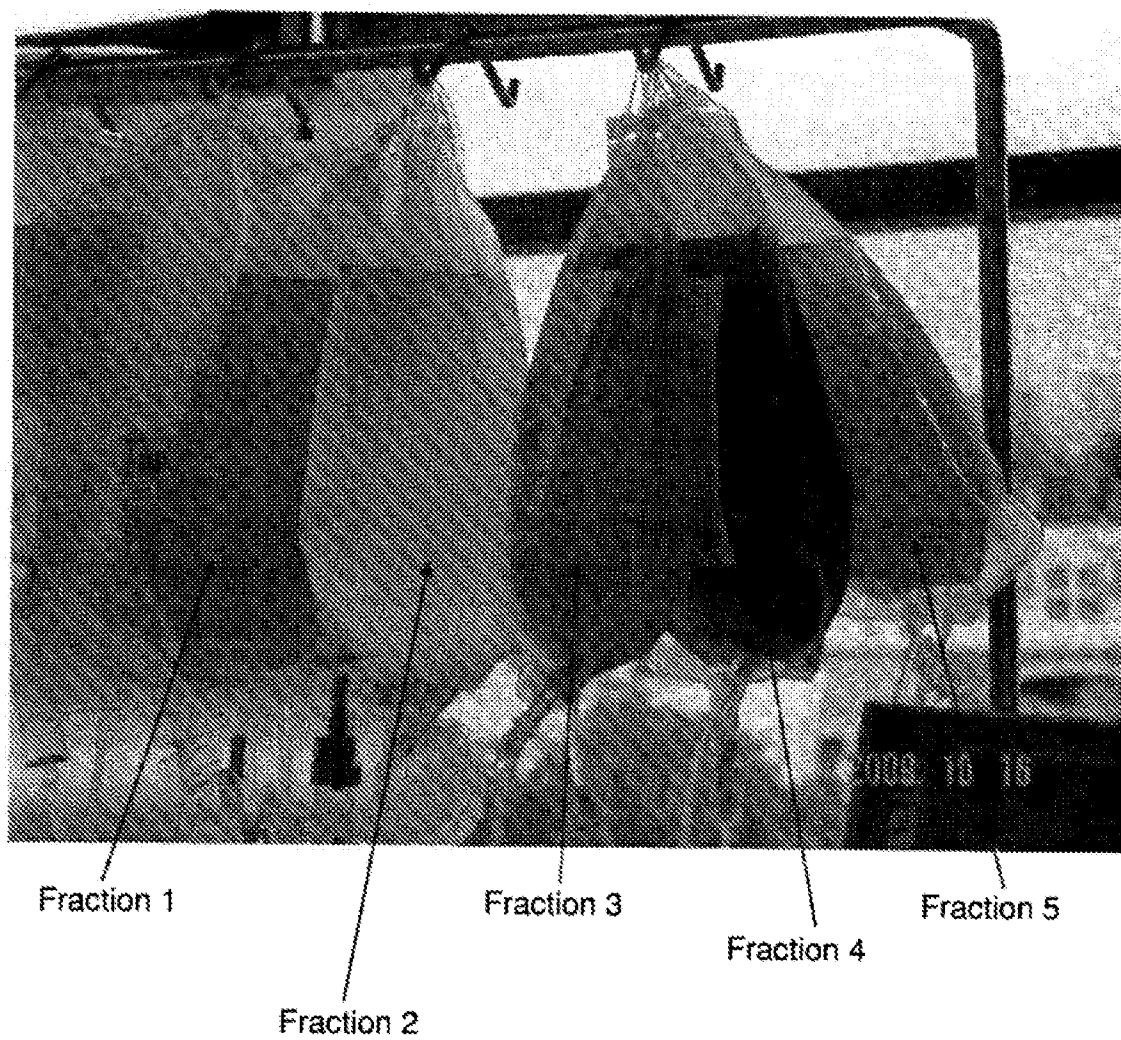


FIGURE 3

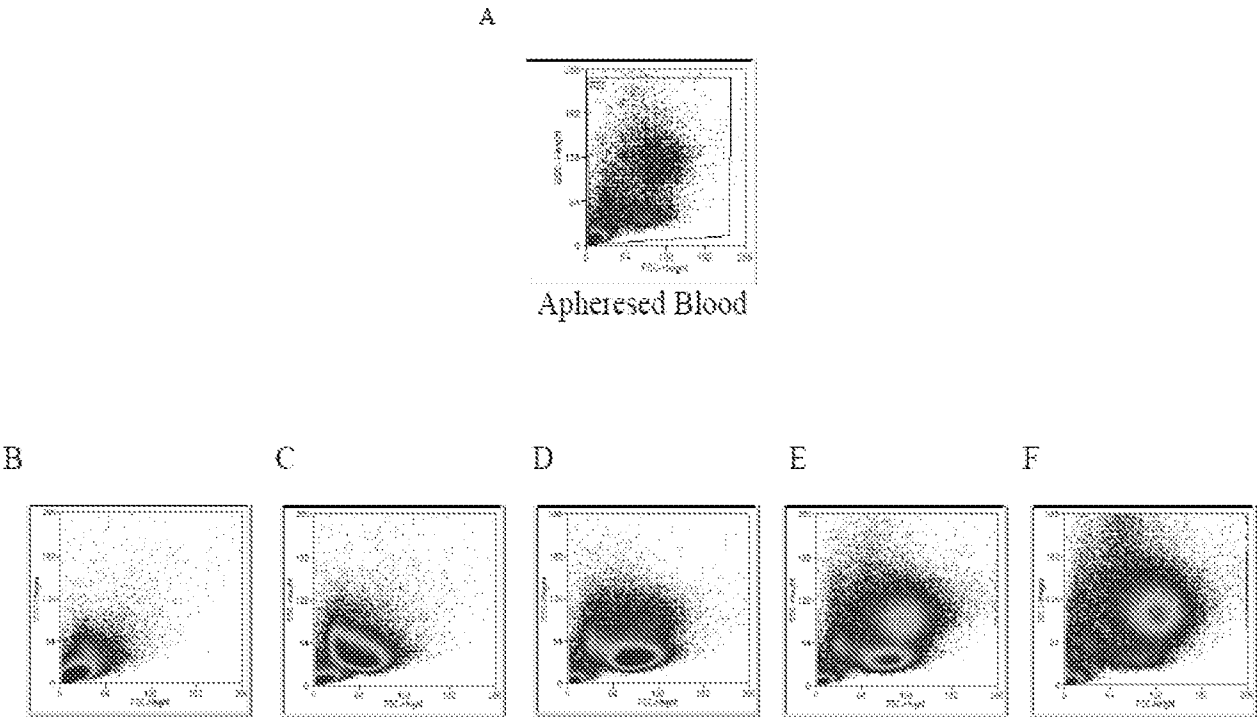


FIGURE 4

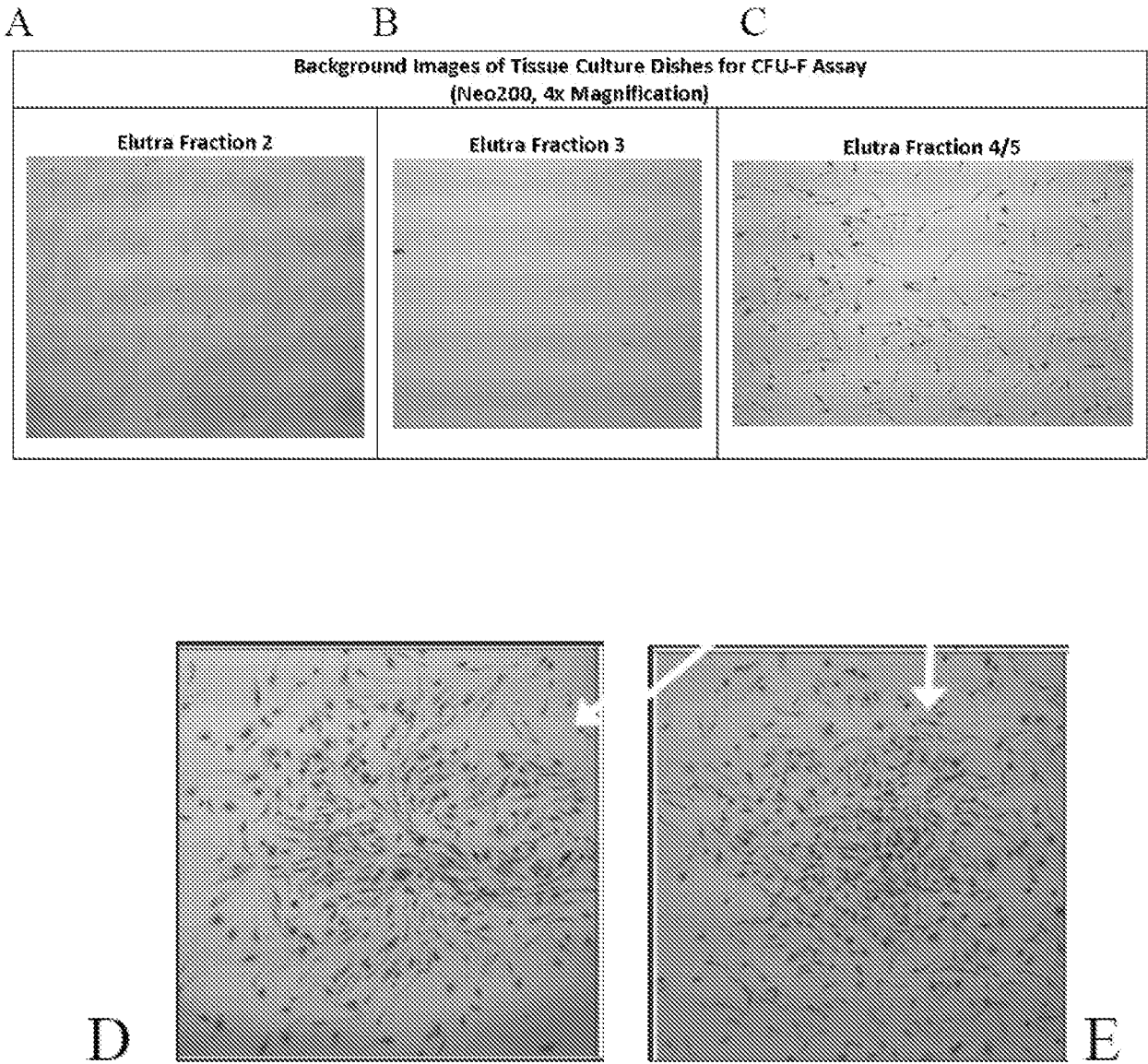


FIGURE 5

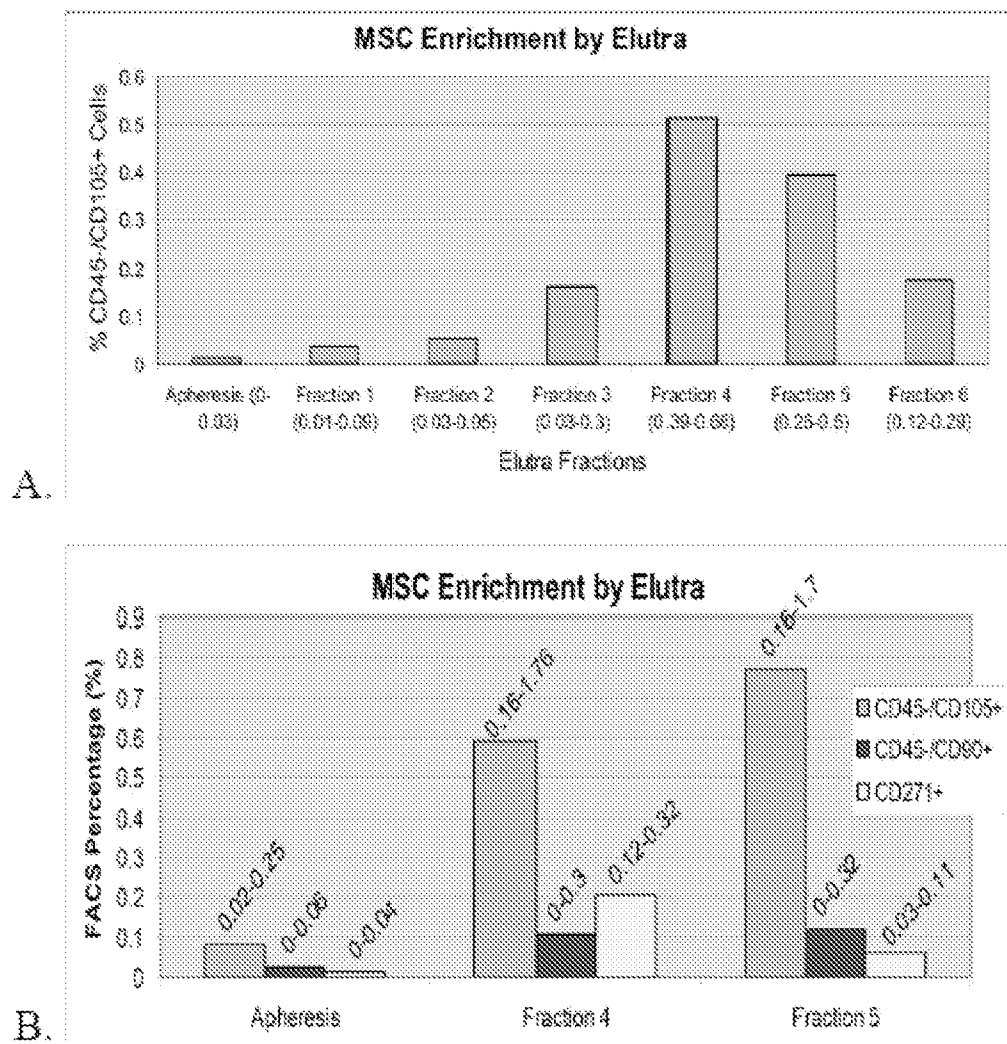
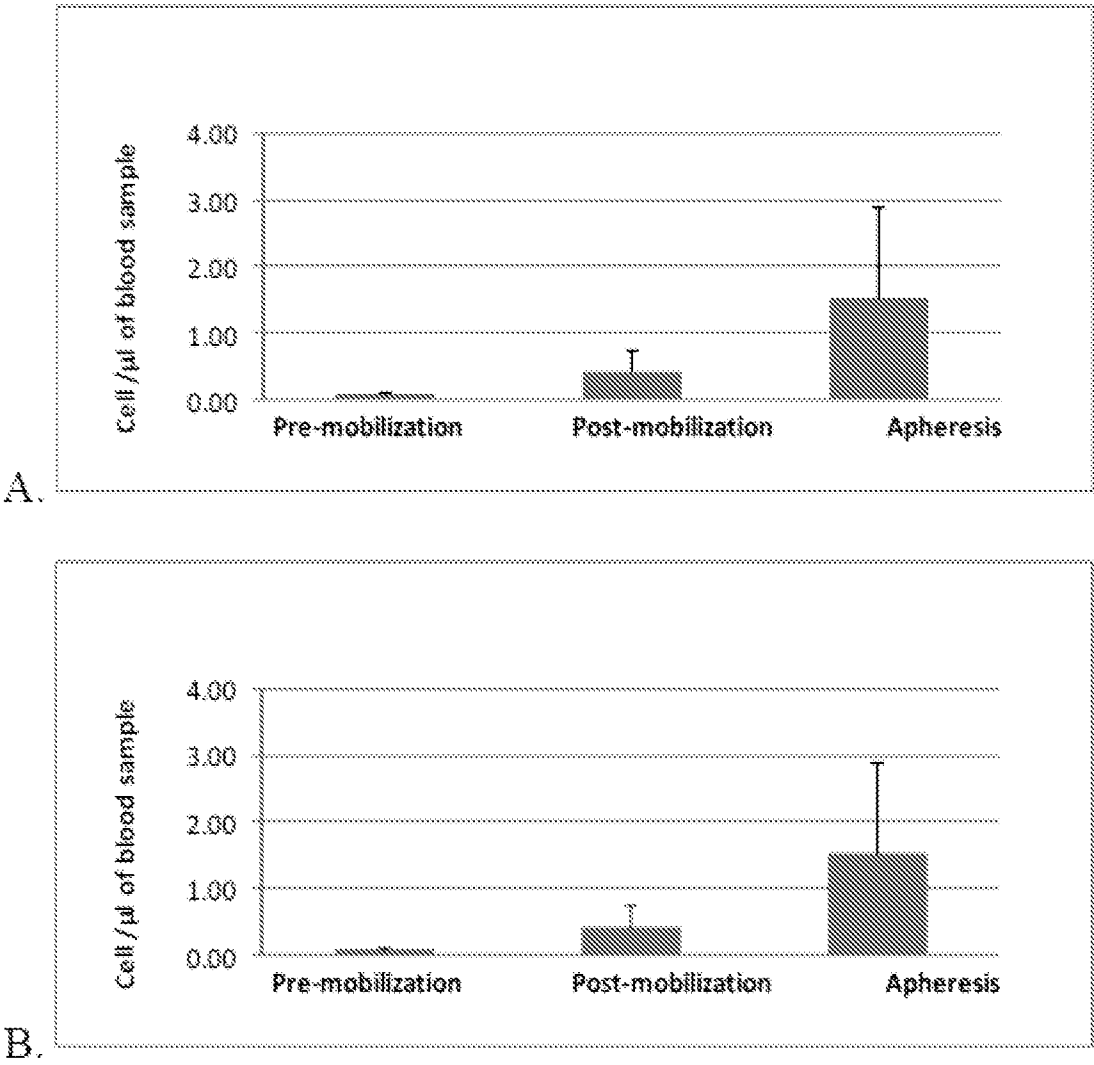


FIGURE 6



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 10/58974

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A01N 1/02 (2010.01)

USPC - 435/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A01N 1/02 (2010.01)

USPC - 435/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
435/372.1, 372.2, 372.3, 325; 210/782

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST - DB=PGPB,USPT,USOC,EPAB,JPAB; PLUR=YES; OP=ADJ; Google Scholar

search terms: cell, blood, particle, sort\$, isolat\$, separat\$, fractio\$, elutriat\$, select\$, fraction, subpopul\$, peripheral, rate, flow, fluid, flow\$, fluid\$, elut\$, Very Small Embryonic Like, mesench\$, hematop\$, progenit\$, stem, pluripot\$, multipot\$, msc, hsc, hct, %, percent,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2005/0250204 A1 (ANTWILER et al.) 10 November 2005 (10.11.2005) para [0021]; [0025]; [0034]; [0035]; [0060]; [0067]; [0076]; [0077]; [0081]; [0084]; [0085]; Fig. 4.	1, 8/1, 20-25, 33/(20-25), 45
----- Y		2-7, 8/(2-7), 26-32, 33/(26-32), 49-51
Y	US 2009/0220466 A1 (RATAJCZAK et al.) 3 September 2009 (03.09.2009) abstract; para [0015]; [0066]; [0103]-[0106]; [0140]; [0181]; [0199].	2, 3, 8/(2,3), 26, 27, 32, 33/(26,27,32), 47-49
Y	US 2007/0095669 A1 (LAU et al.) 03 May 2007 (03.05.2007) para [0024]; [0273]; [0277]-[0279].	4-7, 8/(4-7), 28-32, 33/(28-32), 50, 51
Y	US 2007/0154877 A1 (SCHULER et al.) 05 July 2007 (05.07.2007) para [0012]; [0014]; [0015]; [0017]; [0032]; [0037]; [0044]-[0046].	47, 48



Further documents are listed in the continuation of Box C.



* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

11 January 2011 (11.01.2011)

Date of mailing of the international search report

24 JAN 2011

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

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PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 10/58974

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 9-19, 34-44, and 46
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.