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(54) **Title**: LONG TERM EXPANSION OF HUMAN HEMATOPOIETIC STEM CELLS

(57) **Abstract**: Methods for culturing and increasing the number (expanding the population) of hematopoietic stem cells (HSCs) are provided. In one method, the HSCs are cocultured with mesenchymal stem cells (MSCs) in the presence of angiopoietin-like (Angplt5) growth factor, IGF-binding protein 2 (IGFBP2), stem cell factor (SCF), fibroblast growth factor (FGF), thrombopoietin (TPO), or a combination thereof. HSCs obtained by the methods described can be used in methods to repopulate an animal, such as an immunocompromised animal.

LONG TERM EXPANSION OF HUMAN HEMATOPOIETIC STEM CELLS

RELATED APPLICATION(S)

This application claims the benefit of U.S. Provisional Application No. 61/217,344, filed on May 29, 2009.

The entire teachings of the above application(s) are incorporated herein by reference.

GOVERNMENT SUPPORT

The invention was supported, in whole or in part, by a grant AI069208 from the National Institutes of Health/ National Institute of Allergy and Infectious Diseases (NIAID). The Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Hematopoietic stem cells (HSCs) are multipotent stem cells that give rise to the blood cell types including myeloid (*e.g.,* monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and lymphoid lineages (*e.g.,* T-cells, B-cells, NK-cells). Proliferation and differentiation of hematopoietic stem cells produces the cells found in the hematopoietic system.

Because of the multipotency of HSCs, these are attractive cells for use in disease treatment. It is desirable to have access to large amounts of HSCs. However, HSCs are only present in extremely low numbers in certain tissues, such as bone marrow and umbilical cord blood. Therefore, there is a need for improved methods of expanding HSCs, wherein the HSCs retain their stem cell multipotency.

SUMMARY OF THE INVENTION

Described herein are methods for culturing and increasing the number (expanding the population) of hematopoietic stem cells (HSCs). One aspect of the invention is a method for expanding a population of HSCs comprising co-culturing the HSCs with mesenchymal stem cells (MSCs) in the presence of angiopoietin-like 5 (Angplt5) growth factor, IGF-binding protein 2 (IGFBP2), stem cell factor (SCF), fibroblast growth factor (FGF), thrombopoietin (TPO), or a combination thereof, to produce a cell culture. The cell culture is maintained under conditions in which an expanded population of HSCs is produced.

Another aspect of the invention is a method for expanding a population of HSCs, wherein the method selectively inhibits the expansion of HSCs that differentiate into CD3+ cells when transplanted into a mammal. The method comprises co-culturing HSCs with MSCs in the presence of an inhibitor of Activin A, to produce a combination. The combination is maintained under conditions in which an expanded population of HSCs are produced, and wherein the expanded population of HSCs when transplanted into a mammal differentiate into fewer CD3+ cells as compared to an expanded population of HSCs co-cultured with MSCs is the absence of an inhibitor of Activin A.

Another aspect of the invention is a method for expanding a population of HSCs comprising co-culturing a seed population of HSCs with MSCs, wherein the MSCs are genetically engineered to express angiopoietin-like 5 (Angplt5) growth factor, thereby producing a cell culture. The cell culture is then maintained under conditions in which the population of HSCs is expanded 50 fold or more, as compared to the seed population of HSCs.

Another aspect of the invention is the expanded population of HSCs produced by the methods described herein.

In another aspect, the methods further comprise transplanting the expanded population of HSCs into a mammal.

Another aspect of the invention is a mammal produced by the methods described herein.

Another aspect of the invention is a method for expanding a population of HSCs comprising culturing a seed population of HSCs in the presence of three or more growth factors selected from the group consisting of angiopoietin-like 5 (Angplt5) growth factor, IGF-binding protein 2 (IGFBP2), stem cell factor (SCF), fibroblast growth factor (FGF), and thrombopoietin (TPO), to produce a cell culture. The cell culture is maintained under conditions in which the number of HSCs is expanded at least 50 fold as compared to the number of HSCs present in the seed population of HSCs.

HSCs produced by the methods of the invention are capable of being used in a variety of applications, including transplantation, sometimes referred to as cell-based therapies or cell replacement therapies, such as bone marrow transplants, gene therapies, tissue engineering, and *in vitro* organogenesis. Such applications are useful for the treatment of various disorders, for example, hematological disorders, such as cancers (*e.g.*, leukemia, lymphoma), anemias (*e.g.*, aplastic anemia, sickle cell anemia, Fanconi anemia), lymphocytopenia, neutropenia, thrombocytopenia, *etc.* Thus, the present invention is also directed to methods of treating an individual comprising administering hematopoietic stem cells to the individual. In one embodiment, the method comprises providing HSCs from the individual or from a donor, culturing the HSCs as described herein, and introducing or transplanting the cultured HSCs into the individual. The present invention also provides methods of treating an individual in need of a hematopoietic stem cell-based therapy, comprising providing HSCs from the individual or from a donor; culturing the HSCs as described herein, harvesting the cultured HSCs, and transplanting the cultured HSCs into the individual.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be provided by the Office upon request and payment of the necessary fee.

FIG. 1 is a series of photographs of cord blood HSCs cultured with MSCs (either MSC-GFP – control, or MSC-Angptl5) at Day 3 (D3), Day 5 (D5), Day 7 (D7) and Day 11 (D11).

FIGS. 2A and 2B are graphs. FIG. 2A is a graph of total nuclear expansion of HSCs in different conditions. FIG. 2B is a graph of fold increase in $CD34^+$, $CD133^+$ cells under different conditions. As used herein, "Standard Exp." refers to cell free conditions.

FIGS. 3A and 3B illustrate the percentage of HSCs when cultured over time. FIG. 3A is a series of FACS scans for CD34+ and CD133+ cells under different conditions at Day 6, Day 11 and Day 14. FIG. 3B is a graph charting the percentage of HSCs in culture over time. As used herein, "Cell-free CT" or "FCF" refers to cell free conditions or feeder cell free conditions, and "MSC CT" refers to the MSC control, MSC-GFP.

FIGS. 4A and 4B are graphs illustrating cell-to-cell contact improves *ex vivo* proliferation of HSCs. In FIG. 4A, the absolute number of cell proliferation regardless of their status (differentiated, progenitors or stem cells) is presented. In FIG. 4B only stem cell proliferation is presented (not differentiated cells). FIGS. 4A and 4B: Purified $CD34^+CD133^+$ cord blood cells were co-cultured with MSC-A5 or MSC-GFP with SCF, TPO, FGF and IGFBP2 added in the media, or cultured under the FCF condition, or co-cultured with MSC-A5 in transwells (MSC-A5 TW) with the four growth factors, or co-cultured with MSC-GFP with the four growth factors plus recombinant Angplt5 (MSC-GFP + A5). At the indicated days after culture, hematopoietic cells were removed from the cultures by gentle pippeting, counted, and analyzed for CD34 and CD133 expression by flow cytometry. FIG. 4A: Total numbers of hematopoietic cells in the different conditions during the course of the culture. FIG. 4B: Total number of $CD34^+CD133^+$ cells in the different conditions during the course of the culture.

FIG. 5 is a bar graph illustrating HSCs cultured on MSCs in serum-free conditions and in the presence or absence of growth factors as indicated. The y-axis is a percentage of HSC proliferation normalized to MSCA5. $CD34^+CD133^+$ cord blood cells were co-cultured with MSC-A5 or MSC-GFP without or with addition of the indicated growth factors for 11 days. Hematopoietic cells from the cultures were analyzed as described herein. The expansion of DP cells in different cultures was normalized to that under the standard condition where $CD34^+CD133^+$ cord blood cells were co-cultured with MSC-A5 in the presence of SCF, TPO, FGF and

IGFBP2 in the media. Shown are relative percentages of DP cells at day 11 of culture under the different conditions.

FIG. 6 is a graph illustrating long-term *ex vivo* expansion of HSCs cultured with MSCs (either MSC-GFP – control, or MSC-Angptl5).

FIGS. 7A-7C illustrate the results of short-term repopulating engraftment of HSCs. FIG. 7A outlines the experimental time line. After 10 days of culture, 10^5 human stem/progenitor cells ($CD34^+$ $CD133^+$) were transplanted into sublethally irradiated NOD/SCID gamma chain newborn mice. Human cell engraftment in the blood of the recipients was determined 14 weeks later. Mice injected with cells cultured with or without the presence of feeder cells showed a similar rate of human CD45 in the blood cells. At 14 weeks post-transplantation, mice were euthanized and assessed for the presence of human cells in the bone marrow and different lymphoid organs: blood (FIG. 7B) and spleen (FIG. 7C). The most striking difference observed when comparing both groups is the presence of large number of CD3+ cells in all organs of mice injected with HSC from MSCA5 (also referred to herein as MSC-Angptl5) culture.

FIGS. 8A-8B illustrate the results of long-term ex vivo expansion of HSC supported by MSCs expressing Angptl5. HSCs cultured for a longer time on MSCs layer expressing Angptl5 do not lose their homing and reconstitution abilities as they show similar engraftment percentage as short-term cultured cells (FIG. 8A). Moreover , they present similar percentage of CD3, CD33, CD66b, CD14 positive cells when compared to cells that were cultured in same conditions for shorter time (11 days) (see FIG. 8B). Each bar of FIG. 8B represents the results from a single mouse.

FIGS. 9A and 9B illustrate experimental data of stem cell expansion and purification using methods as described herein. FIG. 9A is a bar chart quantifying expansion of HSCs from cord blood and adult peripheral blood in cell free culture and MSC co-culture with bone marrow or embryonic stem cell derived MSCs. FIG. 9B illustrates flow cytometric analysis of CD133+CD34+ stem cells in unmobilized adult peripheral blood and trial CD34+ positive selection showing purification of CD133+ CD45 low HSCs from peripheral blood. Numbers indicate % of total cells within the gate.

FIGS. 10A-10E show that a combination of co-culture and growth factors enhance expansion of $CD34^+CD133^+$ cord blood cells. Purified $CD34^+CD133^+$ cord blood cells were co-cultured with MSC-A5 or MSC-GFP in the presence of SCF, FGF, IGFBP2, TPO and heparin or cultured in the FCF culture. FIG. 10A: Co-culture with MSC-A5 was visualized at indicated time points by an inverted microscope (10x magnification). (FIGS. 10B-10E) Non-adherent cells in MSC-A5 and MSC-GFP co-cultures and FCF culture were harvested at 7, 11, and 14 days, enumerated, and stained with for CD34 and CD133 antibodies and followed by flow cytometry analysis. FIG. 10B: Representative CD34 versus CD133 staining profiles of cultured cells. The number indicates the percentage of double positive (DP) cells (also referred to herein as DPC) in the gated region. FIG. 10C: The average of percentages of DP cells in the cultures at different time points from three experiments. FIG. 10D: Fold increase of total hematopoietic cells during the course of the cultures. FIG. 10E: Fold increase of $CD34^+CD133^+$ cells during the course of the cultures. The experiments were repeated at least six times and representative data are shown.

FIGS. 11A-11F demonstrate that expanded cells are capable of differentiating into multiple lineages of blood cells in NSG mice. FIG. 11A: Ten days after expansion in the MSC-A5 or FCF cultures, expanded cells containing 10^5 DP cells, were injected into newborn NSG pups. Fourteen weeks after injection, the presence of various lineages of human blood cells in the blood, spleen and bone marrow were analyzed by flow cytometry. Except for mouse CD45 (mCD45) versus human CD45 (hCD45) staining profiles, which were gated on total live cells from the individual tissues, all other staining profiles were gated on human $CD45^+$ cells. The number indicates the percentages of positive cells in the gated region, except the percentages of human or mouse $CD45^+$ cells, which was calculated by dividing the percentage of human or mouse $CD45^+$ cells with the sum of percentages of human and mouse $CD45^+$ cells. Representative dot plots are shown from mice reconstituted with three different cord blood cells. FIG. 11B: Blood was sampled at 8 and 12 weeks post-engraftment and mononuclear cells were stained for human CD45, CD3 and CD19. The percentages of $CD3^+$ cells among $CD45^+$ cells are shown for mice reconstituted with expanded cells from MSC-A5 co-cultures or FCF culture. Each

symbol represents one mouse. The horizontal bar indicates the median value. FIGS. 11C-11F: Comparison of H&E, or CD3 and CD20 immunohistochemical staining of spleen sections of NSG mice (FIG. 11C and FIG. 11E) and NSG mice engrafted with expanded cells from MSC-A5 co-cultures (FIG. 11D and FIG. 11F, 20 wks after engraftment). (FIG. 11C, FIG. 11D) H&E staining. Magnification 4x. (FIG. 11E, FIG. 11F) Anti-CD3 and anti-CD20 staining. Scale bar is 100μm.

FIGS. 12A-12C demonstrate that expanded cells are capable of stable long-term and efficient secondary reconstitution. FIG. 12A: Stable long-term reconstitution by expanded cells. Day 11-expanded cells from either MSC-A5 co-culture or FCF culture were engrafted into sublethally irradiated newborn pups (10^5 DP cells per recipient). Mice were bled at the indicated time points and PBMCs were analyzed for human CD45 and mouse CD45. Reconstitution in the spleen was also assayed in some mice 24 weeks after engraftment. Dot plots show mCD45 versus hCD45 staining profiles of PBMCs at 14 and 24 weeks, or spleen at 24 weeks of the same mouse engrafted with either expanded cells from co-culture or FCF culture. FIG. 12B: Comparison of percentages of human CD45 cells in PBMCs of mice reconstituted with expanded cells from MSC-A5 co-culture. One symbol represents one mouse. The same symbol represents the same mouse at different time points. FIG. 12C: Serial transfer. Day 11-expanded cells from MSC-A5 co-culture was transferred into irradiated newborn pups (10^5 DP cells per recipient). Fourteen weeks later, bone marrow cells were harvested from primary mice and human $CD34^+$ cells were enriched by magnetic sorting and transferred into sublethally irradiated newborn pups. Twelve weeks after secondary transfer, the presence of human $CD45^+$ cells in the bone marrow was analyzed by flow cytometry. mCD45 versus hCD45 staining profile is shown for a representative secondary recipient mouse. The experiments were done twice with expanded cells from two different cord blood sources.

FIGS. 13A-13C demonstrate that expanded DP cells have the same repopulation capacity as unexpanded cells. (FIG. 13A-13B) Limiting dilution assay. $CD34^+CD133^+$ cord blood cells were expanded by co-culture or FCF culture for 11 days and DP cells were purified by cell sorting (95% purity). 5000, 1000, or 400 unexpanded $CD34^+CD133^+$ cells or the same number of expanded cells from the

same cord blood donor were transferred into irradiated newborn pups. A non-toxic green food dye was mixed with cell solutions just before the injection in order to monitor whether the intracardiac injection was successful as the pups turned green if the injection was successful. Mice with failed injections were excluded from analysis. Eight weeks later, mice were analyzed for human CD45 cells in the bone marrow. FIG. 13A: Comparison of percentages of human $CD45^+$ cells in the bone marrow of recipient mice injected with different numbers of unexpanded or expanded DP cells. Mice were considered as non-chimera if the percentage of human $CD45^+$ cells in the bone marrow is below 0.05%. The data were from one of three experiments. Each symbol represents one mouse. FIG. 13B: Limiting dilution analysis for estimating the frequency of SRCs. The numbers shown indicate the calculated frequency of SRCs using the maximum likelihood estimator. FIG. 13C: Comparison of unexpanded and expanded DP cells in competitive repopulation assay. $CD34^+CD133^+$ cord blood cells were purified from a HLA.$A2^+$ and a HLA-$A2^-$ cord blood. A portion of the cells from each donor was expanded on MSC-A5 co-culture for 10 days and $CD34^+CD133^+$ cells purified. Unexpanded or expanded cells from HLA-$A2^+$ donor were mixed in equal proportion (5×10^4 from each) with unexpanded or expanded cells from HLA-$A2^-$ donor and transferred into irradiated newborn pups. Eight weeks after injection, PBMCs were analyzed for human CD45 and HLA-A2 by flow cytometry. The average percentages of HLA-A2+ cells among human $CD45^+$ cells are shown (n=5 per group).

FIGS. 14A-14B demonstrate that MSCs modulate lymphopoiesis through activin A. FIG. 14A: $CD34^+CD133^+$ cord blood cells were expanded in FCF culture for 11 days in the presence or absence of activin A (500 ng/ml). Expanded cells were engrafted into irradiated pups (10^5 DP cells). Ten weeks later, PBMCs were analyzed for human CD45, CD3 and CD19 by flow cytometry. Percentages of $CD3^+$ cells and $CD19^+$ cells among human $CD45^+$ cells were calculated and shown for each mouse. Representative data from one of three independent experiments are shown. FIG. 14B: The experiment was done as in FIG. 14A, except MSC-A5 co-culture was used to expand $CD34^+CD133^+$ cord blood cells and follistatin (FLS) was used instead of activin A. Percentages of $CD3^+$ cells and $CD19^+$ cells among human

$CD45^+$ cells were shown for each mouse. Representative data from one of two independent experiments are shown.

FIGS. 15A and 15B demonstrate T cell responses in mice engrafted with co-culture expanded cells. $CD34^+CD133^+$ cord blood cells were expanded for 11 days in either the co-culture or the FCF culture and injected into sublethally irradiated newborn pups. Sixteen weeks later, mice were screened for levels of reconstitution in the peripheral blood. Mice with similar human leukocyte reconstitution (20-55%) were immunized with tetanus toxoid (TT) three times with 3 weeks interval. Two weeks after the third immunization, spleens were harvested and the percentages of human T cells were determined by flow cytometry. For ELISPOT assay, the same number ($5x10^5$) of human T cells from different samples were seeded into wells coated with anti-human IFN-γ antibody and cultured for 48hrs under three conditions: medium alone (control), in the presence of PMA or in the presence of a TT-specific peptide. ELISPOT was developed and counted. FIG. 15A: Representative ELISPOT wells with splenocytes from immunized mice. FIG. 15B: Comparison the numbers of IFN-γ immunospots among different samples. Each symbol represents one mouse. Data shown are from one of two independent experiments.

FIG. 16 is a table comparing reconstitution levels in NSG recipient mice engrafted with expanded cells from either the MSC-A5 co-cultures or the FCF cultures. See FIG. 11A legend for detailed description of experimental procedures and analysis. Shown are the median percentages and the range of the percentages of different human blood lineage cells in the blood, spleen and bone marrow (BM). Data were obtained from analysis of 9 and 3 mice engrafted with expanded cells from the MSC-A5 co-culture and the FCF culture, respectively. Representative data from one of the two sets of analyses are shown.

FIGS. 17A and 17B are graphs. Purified CD34+CD133+ cord blood cells were co-cultured with MSC-A5 or MSC-GFP in the presence of SCF FGF, IGFBP2, TPO and heparin or cultured in the FCF culture. FIG. 17A: non-adherent cells in MSC-A5 and MSC-GFP co-cultures and FCF culture were harvested at 7, 11 and 14 days, enumerated and stained for CD34 and followed by flow cytometry analysis. The percentages of DP cells in the cultures at the different time points are graphed.

FIG. 17B: Fold increase of CD34+CD133+ (DP) cells during the course of the cell cultures.

FIG. 18 graphs the results of comparing HSC expansion in cell-free medium and MSC co-cultures. HSCs isolated from cord blood (CB-HSCs) and adult peripheral blood (PBMC-HSCs) were cultured in cell-free medium or with angiopoietin like-5 expressing MSCs from bone marrow (BM-MSC-A5A) or derived from ES cells (ES-MSC-A5) for 11 days. Cells in the culture were counted and assayed for CD34 and CD133 expression. Fold increase of CD34+CD133+ HSCs as compared to input cell numbers are shown.

DETAILED DESCRIPTION OF THE INVENTION

Hematopoietic stem cells (HSCs) give rise to all blood lineage cells, which function in diverse biological processes from immune responses to oxygen transport. Transplantation of HSCs is widely used to treat congenital immunodeficiencies and as part of the treatment of hematologic cancers. Bone marrow, mobilized peripheral blood and umbilical cord blood are the major sources of HSCs for transplant and research. However, both research and treatment are often limited by the availability of HSCs from suitable matching donors as well as absolute cell number available from any single donor. This is especially true of umbilical cord blood samples which by their nature contain a very small population of HSCs. Consequently, robust methods for a long term HSCs expansion *ex vivo* are needed.

To date, others have reported HSC *ex vivo* cultures produce a 20-fold expansion obtained after 11 days of culture with a cocktail of growth factors (Zhang, *et al.*, Blood (2008) 111: 3415-23). After that time, the cells entered a senescence stage, which prevented further expansion of these cells. Furthermore, this limited expansion has a very high cost if used at a larger scale, particularly due to the high price of the growth factors such as Angptl5 required for the culture.

Previous clinical trials using *ex vivo*-expanded cells in human bone marrow transplantation have failed to show significantly improved hematopoietic recovery (Dick *et al.*, Stem Cells Vol. 26 No. 10 October 2008, pp. 2552 -2563). The creation of an *ex vivo* expansion model that mimics the physiological "micro-environment" of hematopoietic stem cells (HSCs) in the bone marrow could

overcome this hurdle and translate directly into increased clinical applications of cord blood for clinical applications, such as bone marrow transplantation, as well as research applications. Thus, increasing the number of HSCs available for use in bone marrow transplantation (such as supplementing or replacing the need for bone marrow transplant, *e.g.*, using cord blood as the source of HSCs to replace bone marrow transplant, and/or using HSCs from bone marrow to supplement a bone marrow transplant), in cell-based therapy, for gene delivery and expression in a recipient of the cells, and the like, is desired.

Described herein are methods for expanding a population of hematopoietic stem cells (HSCs) *in vitro* or *ex vivo*. By "expanding a population" it is meant that the population of cells (*e.g.*, HSCs) are increased, or multiplied, as compared to the number of cells originally seeded into the culture (sometimes referred to herein as the "seed population" of cells), by maintaining the cells under conditions appropriate for cell division of the cells, thereby increasing or expanding the population of cells. The increase in cell number is commonly referred to by those of skill in the art as a "fold increase" when comparing the starting number of cells to the expanded or increased number of cells. For example, a "10 fold" increase means the cells have increased to ten times the original number of cells (*e.g.*, in the seed population).

This new technology also has wider applications beyond bone marrow transplants, such as cell targets for hematopoietic gene therapy, for use in further experimental research as well as a research tool, *e.g.*, in research to gain further understanding of the mechanisms of stem cell regeneration, maturation, and differentiation, gene expression analyses, profiling, *etc.* Also, lowering the cost of HSC expansion will make the technology affordable for many research applications such as the creation of healthy or disease humanized mice models.

Thus, one aspect of the invention is a method for expanding a population of HSCs comprising culturing HSCs in the presence of one or more, two or more, three of more, four or more, or five or more growth factors. In one embodiment, the growth factors added to the co-culture are angiopoietin-like 5 (Angplt5) growth factor, IGF-binding protein 2 (IGFBP2), stem cell factor (SCF), fibroblast growth factor (FGF), thrombopoietin (TPO), or any combination thereof. As will be appreciated by one of skill in the art, other components can be added to the cell

culture medium. In one embodiment, the method for expanding a population of HSCs comprises adding heparin to the cell culture.

As used herein, HSCs are multipotent stem cells that give rise to the blood cell types including myeloid (*e.g.*, monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and lymphoid lineages (*e.g.*, T-cells, B-cells, NK-cells). HSCs express the cell marker CD34, and are commonly referred to as "CD34+". As understood by those of skill in the art, HSCs can also express other cell markers, such as CD133 and/or CD90 ("CD133+", "CD90+"). In some instances, HSCs are characterized by markers that are not expressed, *e.g.*, CD38 ("CD38-"). Thus, in one embodiment of the invention, the population of HSCs that are expanded and used in the methods described herein are CD34+, CD90+, CD133+, CD34+CD38-, CD34+ CD90+, CD34+CD133+CD38-, CD133+CD38-, CD133+CD90+CD38-, CD34+CD133+CD90+CD38-, or any combination thereof. In a particular embodiment, the HSCs are both CD34 ("CD34+") and CD133+ ("CD133+"), also referred to herein as "double positive" or "DP" cells or "DPC". In another embodiment, the HSCs are CD34+CD133+ and CD38- and/or CD90+.

HSCs are found in bone marrow such as in femurs, hip, ribs, sternum, and other bones of the donor (*e.g.*, vertebrate animals such as mammals, including humans, primates, pigs, mice, *etc.*). Other sources of HSCs for clinical and scientific use include umbilical cord blood, placenta, fetal liver, mobilized peripheral blood, non-mobilized (or unmobilized) peripheral blood, fetal liver, fetal spleen, embryonic stem cells, and aorta-gonad-mesonephros (AGM), or a combination thereof.

As will be understood by persons of skill in the art, mobilized peripheral blood refers to peripheral blood that is enriched with HSCs (e.g., CD34+ cells). Administration of agents such as chemotherapeutics and/or G-CSF mobilizes stem cells from the bone marrow to the peripheral circulation. For example, administration of granulocyte colony-stimulating factor (G-CSF) for at least, or about 5 days mobilizes CD34+ cells to the peripheral blood. A 30-fold enrichment of circulating CD34+ cells is observed with peak values occurring on day 5 after the start of G-CSF administration. Without mobilization of peripheral blood, the

number of circulating CD34+ cells is very low, estimated between 0.01 to 0.05% of total mononuclear blood cells. Typically liters of blood are required to obtain a small fraction of CD34+ cells. However, using the methods disclosed herein, in particular the expansion protocol, even if starting with a low number of CD34+ cells in a peripheral blood sample, such as a non-mobilized blood sample, CD34+ cells increase at least about a 1000 fold.

As known in the art, HSCs can be obtained from these sources in a variety of ways. For example, HSCs can be obtained directly by removal from the bone marrow, e.g., in the hip, femur, *etc.*, using a needle and syringe, or from blood following pre-treatment of the donor with cytokines, such as granulocyte colony-stimulating factor (G-CSF), that induce cells to be released from the bone marrow compartment.

In one embodiment, the HSCs are obtained from a single donor. In another embodiment, the HSCs used in the methods described herein are freshly isolated HSCs, cryopreserved HSCS, or a combination thereof. In one embodiment, the HSCs are human HSCs (in other words, obtained from a human donor).

It has also been discovered that cell-to-cell contact between HSCs and mesenchymal stem cells (MSCs), in the presence of growth factors (*e.g.*, IGF-binding protein 2 (IGFBP2) and angiopoietin-like 5 (Angptl5)), act synergistically to markedly expand the population of HSCs in culture. For example, culturing HSCs on a MSC layer expressing Angptl5 produced a remarkable 64-fold expansion of HSCs in the same period of culture as prior reports (11 days). Importantly, the HSCs maintain their stem cell characteristics while producing this high rate of proliferation to obtain an impressive fold expansion of HSCs. As described herein, long term culture of HSCs for more than 40 days which increased HSCs expansion to an impressive 26,100 fold was successfully accomplished.

As used herein, MSCs are multipotent stem cells that can differentiate into a variety of cell types, *e.g.*, osteoblasts, chondrocytes, myocytes, adipocytes. MSCs are found in bone marrow, umbilical cord blood, umbilical cord tissue, fetal tissue, embryonic stem cells, etc. from a donor. MSCs can be obtained using methods known in the art (*e.g.*, derived from embryonic stem cells, Lian *et al.*, Stem Cells. (2007) 25(2):425-436), bone marrow, umbilical cord blood, umbilical cord tissue,

fetal tissue, or a combination thereof), or from commercial sources. In one embodiment, the MSCs used in the methods described herein are freshly isolated MSCs, cryopreserved MSCs, or a combination thereof. In another embodiment, the MSCs are obtained from a mammal, *e.g.*, a human, primate, pig, mouse, and the like. In a particular embodiment, the MSCs are human MSCs (in other words, obtained from a human donor). In another embodiment, the MSCs are obtained from a single donor. In yet other embodiments, the HSCs and MSCs are obtained from the same donor or are obtained from different donors.

As described herein, the HSCs and MSCs are combined in a co-culture. As will be appreciated by those of skill in the art, the ratio of HSCs to MSCs in co-culture can vary and need not be exact. Furthermore, the ratio of HSCs to MSCs will vary after initial seeding of the cells due to varying cell division cycles of the cells in co-culture. In one embodiment, the ratio of HSCs to MSCs (HSCs:MSCs upon initial seeding of the cells) is at least, or about 40:1, at least, or about 20:1, at least, or about 10:1, at least, or about 1:1, at least, or about 1:5, at least, or about 1:10, at least, or about 1:20, or at least, or about 1:40. In particular embodiments, the ratio of HSCs to MSCs is , at least, or about 1:2, at least, or about 1:3, at least, or about 1:4, at least, or about 1:5

As also described herein, one aspect of the invention is a method for expanding a population of HSCs comprising co-culturing HSCs with mesenchymal stem cells (MSCs) in the presence of one or more, two or more, three of more, four or more, or five or more growth factors. In one embodiment, the growth factors are angiopoietin-like 5 (Angplt5) growth factor, IGF-binding protein 2 (IGFBP2), stem cell factor (SCF), fibroblast growth factor (FGF), thrombopoietin (TPO), or any combination thereof. As will be appreciated by those skilled in the art, the concentration of growth factors can vary according to the needs. For example, at least, or about 500 ng/ml Angplt5, at least, or about 100 ng/ml IGFBP2, at least, or about 10 ng/ml SCF, at least, or about 10 ng/ml FGF-1, at least, or about 20 ng/ml TPO. In a particular embodiment, the HSCs and MSCs are co-cultured in the presence of at least about 100 ng/ml IGFBP2 (final concentration). The growth factors can be of any species origin, or variant thereof, *e.g.*, human, primate, pig, mouse, *etc.*

As will also be appreciated by those of skill in the art, one or more of the growth factors can be added to the co-culture (or culture) in a variety of ways. In one embodiment, the growth factors are directly added to the co-culture (or culture). In another embodiment, the MSCs and/or HSCs are genetically engineered to express (*e.g.*, synthesize, produce, secrete, surface express, *etc.*) one or more growth factors. As used herein, "genetically engineered to express" means any method in which MSCs and/or HSCs are made to express the desired growth factor. Such methods include, *e.g.*, transfection, transduction, or infection with a suitable vector or expression construct comprising the desired growth factor, genetic recombination, or production of a transgenic animal that expresses the desired growth factor in MSCs (e.g., wherein the MSCs are obtained from such a transgenic animal), and the like. Suitable vectors or expression constructs are well known to those skilled in the art. For example, viral vectors (*e.g.* lentiviral vector, adenovirus vector, retrovirus vector, and the like), or non-viral vectors can be readily designed and used using art standard techniques. Expression of the desired growth factor can be transient, stable, or under controlled expression, as will be appreciated by the person of skill in the art.

In one embodiment, the MSCs are genetically engineered to express angiopoietin-like 5 (Angplt5) growth factor, IGF-binding protein 2 (IGFBP2), stem cell factor (SCF), fibroblast growth factor (FGF), thrombopoietin (TPO), or any combination thereof. In a particular embodiment, the MSCs are genetically engineered to express Angptl5 growth factor. In a further embodiment, the Angptl5 growth factor is human Angptl5 growth factor. In another embodiment, the MSCs are genetically engineered to express IGFBP2. In a further embodiment, the MSCs are genetically engineered to express Angptl5 and IGFBP2. HSCs co-cultured with MSCs engineered to express a growth factor as described herein are able to proliferate and increase the number of HSCs.

The term "co-culturing" is standard in the art and refers to the culture of two or more cell types in the same cell culture vessel or apparatus. As described herein, the cells are maintained under conditions suitable for propagation and expansion of the cell populations. Such suitable conditions include, *e.g.*, suitable temperature (*e.g.*, 37°C), CO_2 concentration, pH, time, suitable vessel or bioreactor (*e.g.*, two-

dimensional devices, such as plates, wells, and three-dimensional devices, such as microcarriers, tubes, lattices, matrices, *etc.*).

In one embodiment, the method of expanding a population of HSCs comprises co-culturing HSCs and MSCs, wherein one or more of the HSCs and one or more of the MSCs are in (direct) cell-to-cell contact. In another embodiment, the co-culture further comprises heparin. In yet another embodiment, the HSCs and MSCs are co-cultured in serum-free conditions (*e.g.*, serum-free medium). In another embodiment, the HSCs and MSCs are co-cultured in defined media conditions. In a further embodiment, the HSCs and MSCs are co-cultured in serum containing media.

In the methods described herein, the HSCs and MSCs can be cultured for any suitable length of time, e.g., a short term culture or a long term culture.

In one embodiment, co-culture of HSCs with MSCs is for at least about 3 days, at least about 4 days, at least about 5 days, at least about 6 days, at least about 7 days, at least about 8 days, at least about 9 days, at least about 10 days, at least about 11 days, at least about 12 days, at least about 13 days, at least about 14 days, at least about 15 days, or more.

In another embodiment, co-culture of HSCs with MSCs is in a method for long-term culture of HSCs. As used herein, "long-term culture" means culture of cells for longer than a more typical 7-11 days. For example, long-term culture is at least, or about 14 days, at least, or about 17 days, at least, or about 21 days, at least, or about 24 days, at least, or about 28 days, at least, or about 32 days, at least, or about 36 days, at least, or about 40 days, at least, or about 44 days, at least, or about 48 days, at least, or about 52 days, at least, or about 56 days, at least about 60 days, at least, or about 64 days, or more. Long-term culture of HSCs results in a marked increase of HSCs from the more typical 7-11 days of culture.

The population of HSCs co-cultured as described herein increases the population of HSCs at least, or about 10 fold, at least, or about 25 fold, at least, or about 50 fold, at least, or about 55 fold, at least, or about 60 fold, at least, or about 65 fold, at least, or about 70 fold, at least, or about 75 fold, at least, or about 80 fold, or more. In one embodiment, the method for culturing (*e.g.*, long-term culturing) of HSCs increases the population of HSCs at least, or about 50 fold, at least, or about

100 fold, at least, or about 150 fold, at least, or about 250 fold, at least, or about 500 fold, at least, or about 1,000 fold, at least, or about 2,500 fold, at least, or about 5,000 fold at least, or about 7,500 fold, at least, or about 10,000 fold, at least, or about 12,500 fold, at least, or about 15,000 fold, at least, or about 17,500 fold, at least, or about out 20,000 fold, at least, or about 25,000 fold, at least, or about 30,000 fold, at least, or about 35,000 fold, or more.

In one embodiment, the expanded population of HSCs is increased about 60 fold in 11 days of co-culture. In another embodiment, the expanded population of HSCs is increased about 150 fold in 14 days of co-culture. In another embodiment, the expanded population of HSCs is increased about 10 fold in 10 days of co-culture, and the HSCs are human HSCs.

As will be appreciated by those skilled in the art, the culture of cells can lead to crowding of the cells in culture (confluence). Using the methods described herein, at least a portion, or even the majority of expanded HSCs can be removed from the culture, and the remaining HSCs in the culture can continue to be expanded in culture. Removal of a portion of the expanded population of cells can achieved by any suitable means, *e.g.*, mechanical means (shaking, scraping, washing, *etc.*). The HSCs not removed from the cell culture vessel remain under conditions suitable for continued expansion of the remaining HSCs. Thus, the method of expanding a population of HSCs, can further comprise removing at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of the expanded population of HSCs and further expanding the remaining HSCs in the co-culture.

The HSCs produced as described herein maintain their stem cell characteristics. Typical characteristics of HSCs is their ability to differentiate into one or more blood cell types. Exemplary blood cell types include phagocytic immune cells (*e.g.*, granulocytes), monocytes (*e.g.*, macrophage precursor cells), macrophages, eosinophils, erythrocytes, platelet forming cells (*e.g.*, megakaryocytes), T lymphocytes, B lymphocytes, and natural killer (NK) cells. Furthermore, HSCs are capable of self-renewal.

In a further embodiment, the expanded population of HSCs comprise severe combined immunodeficient (SCID) mouse repopulating cells (SRCs). SRCs are a sub-population of the HSCs that have severe combined immunodeficient (SCID)

repopulating activity when transplanted into a mammal. In one embodiment, the SRCs are CD34+CD133+. In another embodiment, the SRCs are expanded in the methods described herein at least, or about 80 fold, at least, or about 85 fold, at least, or about 90 fold, at least, or about 95 fold, at least, or about 100 fold, at least, or about 500 fold, about 1,000 fold, at least, or about 2,000 fold, at least, or about 4,000 fold, about 8,000 fold, at least, or about 12,000 fold, or more, within the expanded population of HSCs. In one embodiment, the SRCs are expanded about 80 fold in 11 days of co-culture. In another embodiment, the SRCs are expanded about 12,000 fold in 40 days of co-culture.

MSCs produce endogenous Activin A in cell culture. As shown herein, Activin A secreted by MSCs in the cell culture leads to the expansion of HSCs that are capable of differentiating into CD3+ cells upon transplantation into an animal. Thus, another aspect of the invention is a method of expanding a population of HSCs comprising culturing HSCs in the presence of Activin A, thereby producing an expanded population of HSCs that are capable of differentiating into CD3+ cells upon transplantation into an animal. In one embodiment, the method further comprises culturing the HSCs in the presence of one or more, two or more, three of more, four or more, or five or more growth factors. In one embodiment, the growth factors are angiopoietin-like 5 (Angplt5) growth factor, IGF-binding protein 2 (IGFBP2), stem cell factor (SCF), fibroblast growth factor (FGF), thrombopoietin (TPO), or any combination thereof, as described above. Such an expanded population of HSCs produce an enhanced CD3+ T cell reconstitution in an animal when transplanted, and thus leads to an enhanced T cell response in the animal, *e.g.*, as compared to a suitable control. A suitable control is the T cell response in an animal that was transplanted with cells (*e.g.*, cells derived from the same donor), that were not co-cultured with MSCs or not cultured in the presence of active Activin A. For example, the percentage of T cells in the blood of a mouse transplanted with human HSCs co-cultured with MSCs expressing Angplt5 (MSC-A5) was 45%, as compared to 20% T cells in the blood of a mouse transplanted with human HSCs cultured without MSCs (feeder cell free – FCF) at 12 weeks post- reconstitution.

Yet another aspect of the invention is directed to a method for expanding a population of HSCs, wherein the method selectively inhibits the expansion of HSCs

that differentiate into $CD3^+$ cells when transplanted into a mammal. The method comprises co-culturing HSCs with mesenchymal stem cells (MSCs) in the presence of an inhibitor of Activin A, thereby producing a combination, and maintaining the combination under conditions in which an expanded population of HSCs are produced. The resulting expanded population of HSCs when transplanted into a mammal differentiate into fewer $CD3^+$ cells as compared to an expanded population of HSCs co-cultured with MSCs is the absence of an inhibitor of Activin A. Inhibitors of Activin A are known in the art, *e.g.*, follistatin, follistatin like-3 (FSTL-3), an antagonist antibody (*e.g.*, anti-activin A antibody (IgY) as described by Murata *et al.*, Proc Soc Exp Biol Med. 1996;211(1):100-7), inhibins. Other suitable inhibitors include siRNA, antisense, ribozymes, small molecules, and the like. As described above, the HSCs are expanded under conditions suitable for expansion of the HSCs. In one embodiment, the method further comprises co-culturing the HSCs, MSCs and inhibitor of Activin A in the presence of one or more, two or more, three of more, four or more, or five or more growth factors. In one embodiment, the growth factors are angiopoietin-like 5 (Angplt5) growth factor, IGF-binding protein 2 (IGFBP2), stem cell factor (SCF), fibroblast growth factor (FGF), thrombopoietin (TPO), or any combination thereof, as described above.

In the methods described herein, the expanded population of HSCs can be monitored during or after culture. As described herein, HSCs are CD34+, CD133+, CD34+CD133+ (also referred to herein as CD34+CD133+, or double positive cells "DPC"), or a combination thereof. Thus, in one embodiment, monitoring the expansion of HSCs comprises detecting the expression of CD34, CD133, CD34 and CD133, or a combination thereof by the expanded population of HSCs. Methods for the detection of cells are known in the art, for example, FACS scan flow cytometry, enzyme-linked immunosorbant assay (ELISA), immunostaining, *etc.*

In another embodiment, the methods for expanding HSCs further comprise selecting, isolating or substantially isolating cells before and/or after culture that express CD34 and/or CD133. Methods for the isolation and purification of cells are known in the art. As used herein, "isolated cells" or an "isolated cell population" are cells that are substantially isolated with respect to the complex cellular milieu in which it naturally occurs, or organ, body, or culture medium. In some instances, the

isolated material will form part of a composition (for example, a crude extract containing other substances), buffer system or reagent mix. In other circumstances, the material can be purified to essential homogeneity. An isolated HSC population can comprise at least about 50%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% (on a total cell number basis) of all cells present. In one embodiment, the invention is directed to isolated, or substantially isolated (or purified, substantially purified) HSCs produced by the methods described herein. Thus, in one embodiment is a composition comprising, consisting essentially of, or consisting of HSCs (*e.g.*, a population of expanded HSCs) produced by the methods described herein.

In one embodiment, the methods for producing HSCs additionally or further comprise negatively selecting or isolating cells, before and/or after culture, that express CD38.

Various techniques can be employed to select or isolate the desired cells. For example, the cells can be separated by removing cells of dedicated lineage or having a particular phenotype. Monoclonal antibodies are useful for identifying markers associated with particular cell lineages, stages of differentiation, or particular phenotypes. The antibodies can be attached to a solid support (*e.g.*, a bead, column, well of a plate or dish, and the like) to allow for crude separation. Other methods for isolating HSCs will be appreciated by those of skill in the art, *e.g.*, using enzymatic activity, such as aldehyde dehydrogenase activity (ALDH) as a marker (Hess *et al.*, Blood. 2006:107(5):2162-9).

The separation techniques employed preferably maximizes the retention of viable cells in the fraction to be collected. Various techniques of different efficacy can be employed to obtain relatively crude separations wherein up to about 75%, about 80%, about 85%, or about 90%, but more usually more than about 91%, about 92%, about 93%, about 94%, or about 95% of the total cells have the desired marker in the isolated cell population. In certain embodiments, at least about 96%, about 97%, about 98%, about 99%, or more of the isolated cells have the desired marker. The particular technique employed will depend upon efficiency of separation, associated cytotoxicity, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill.

Procedures for separation can include, but are not limited to, physical separation, magnetic separation, using antibody-coated magnetic beads, affinity chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, including, but not limited to, complement and cytotoxins, and "panning" with antibody attached to a solid matrix, *e.g.*, plate, elutriation or any other convenient technique. In certain embodiments, a high throughput technique is used to rapidly screen and separate different cells.

The use of physical separation techniques include, but are not limited to, those based on differences in physical (density gradient centrifugation and counter-flow centrifugal elutriation), cell surface (lectin and antibody affinity), and vital staining properties (mitochondria-binding dye rho123 and DNA-binding dye Hoechst 33342).

Techniques providing accurate and rapid separation include, but are not limited to, flow cytometry (*e.g.*, fluorescence activated cell sorting "FACS"), which can have varying degrees of sophistication, *e.g.*, a plurality of color channels, low angle and obtuse light scattering detecting channels, impedance channels, *etc.* Cells also can be selected by flow cytometry based on light scatter characteristics, where stem cells are selected based on low side scatter and low to medium forward scatter profiles. Cytospin preparations show the enriched stem cells to have a size between mature lymphoid cells and mature granulocytes.

For example, in a first separation step, anti-CD34 and/or anti-CD133 can be labeled with a first fluorochrome, while the antibodies for the various dedicated lineages, can be conjugated to a fluorochrome with different and distinguishable spectral characteristics from the first fluorochrome. While each of the lineages can be separated in more than one separation step, desirably the lineages are separated at the same time while positively selecting for HSCs. The cells can be selected and isolated from dead cells, by employing dyes associated with dead cells (including but not limited to, propidium iodide (PI)). Other methods can be used as will be appreciated by those of skill in the art. For example, magnetic separation methods (which can be automated) using, either or both, CD34 and CD133 as selection markers.

The cells obtained as described herein can be used immediately or frozen *e.g.,* at liquid nitrogen temperatures, and stored (*e.g.*, for hours, days, weeks, months, or years). The frozen cells can later be thawed and used as desired.

HSCs produced by the methods of the invention are capable of being used in a variety of applications, including transplantation, sometimes referred to as cell-based therapies or cell replacement therapies, such as bone marrow transplants, gene therapies, tissue engineering, and *in vitro* organogenesis. For example, HSCs produced by the methods of the invention are capable of repopulating an animal, *e.g.,* an immunocompromised animal. The animal can be a mammal, *e.g.,* any mammal such as a human, non-human primate, horse, pig, cow, sheep, goat, dog, cat, rat, mouse, *etc*. The method involves administering by standard means, such as intravenous infusion or mucosal injection, the expanded cultured HSCs to the animal.

The expanded HSCs provided by the methods described herein can be used for reconstituting the full range of hematopoietic cells in an immunocompromised host following therapies such as, but not limited to, radiation treatment and chemotherapy. Such therapies destroy hematopoietic cells either intentionally or as a side-effect of bone marrow transplantation or the treatment of lymphomas, leukemia and other neoplastic conditions, *e.g.*, breast cancer. In one embodiment, the animal has a condition including, but not limited to, reduced hematopoietic function, reduced immune function, reduced neutrophil count, reduced neutrophil mobilization, mobilization of peripheral blood progenitor cells, sepsis, severe chronic neutropenia, bone marrow transplant, an infectious disease, leucopenia, thrombocytopenia, anemia, chemical or chemotherapeutic induced bone marrow aplasia or myelosuppression, acquired immune deficiency syndrome, or is in need of enhancing engraftment of bone marrow during transplantation, or enhancing bone marrow recovery in treatment of radiation.

Thus, one embodiment of the invention is a method of reconstituting or repopulating an animal by transplanting an expanded population of HSCs produced by the methods described herein. In one embodiment, the HSCs in the expanded population of HSCs are transplanted into a recipient animal (*e.g.*, a mammal, such as a human, primate, mouse, and the like). In one embodiment, the animal receiving

the expanded population of HSCs is an immunocompromised animal. In another embodiment, the expanded population of HSCs transplanted into a recipient animal gives rise to reconstitution of multiple lineages of blood cells in the animal. By "gives rise to" it is meant that the HSCs differentiate into the multiple lineages of blood cells in the animal (*e.g.*, myeloid progenitor cells, mature hematopoietic cells, or a combination thereof). The multiple lineages of blood cells can be determined by expression of characteristic markers on the cells that differentiate from the expanded population of HSCs. For example, suitable markers include, without limitation, CD3, CD19, IgM, CD14, CD33, CD33, CD34, CD133, CD11c HLA-DR, CD56, CD235a, CD235b, CD235ab, CD41, CD38, CD45RA, CD127, CD90, or a combination thereof. In one embodiment, an immunocompromised animal that receives HSCs develops myeloid progenitor cells derived from the HSCs. As used herein, "derived from" means any cell that is the result of proliferation and/or differentiation of an HSC.

In another embodiment, an animal that receives HSCs develops mature hematopoietic cells derived from the HSCs. In a further embodiment, an animal that receives HSCs develop CD3+ cells derived from the HSCs. CD3 is a pan marker of lymphocyte T cells.

In a particular embodiment, an animal that receives an expanded population of HSCs produced by the methods of the invention as described herein, develop $CD3^+$ cells, $CD19^+$ cells, $CD19^+IgM^+$ cells, $CD14^+CD33^+$ cells, $CD33^+$ cells, $CD34^+$ cells, $CD34^+CD133^+$ cells, $CD11c^+$ $HLA\text{-}DR^+$ cells, $CD56^+$ cells, $CD235a^+$, $CD235b^+$, $CD235ab^+$, $CD41^+$, $CD38^+$, $CD45RA^+$, $CD127^+$, $CD90^+$, or a combination of cells thereof, which are cells differentiated from the expanded population of HSCs.

In another embodiment, the animal that receives an expanded population of HSCs produced by the methods of the invention as described herein develop $CD19^+$ cells, $CD19^+IgM^+$ cells, $CD14^+CD33^+$ cells, $CD33^+$ cells, $CD34^+$ cells, $CD34^+CD133^+$ cells, $CD11c^+$ $HLA\text{-}DR^+$ cells, $CD56^+$ cells, $CD235a^+$, $CD235b^+$, $CD235ab^+$, $CD41^+$, $CD38^+$, $CD45RA^+$, $CD127^+$, $CD90^+$, or a combination of cells thereof, which are cells differentiated from the expanded population of HSCs.

In one embodiment, the reconstitution of multiple lineages of blood cells in the animal is a stable, long term reconstitution. As used herein, "stable, long term reconstitution" means reconstitution of the animal that persists for an extended period of time relative to the lifespan of the animal, as will be appreciated by a person skilled in the art. For example, in mice, long term reconstitution is 12 weeks. Thus, long term reconstitution includes 12 weeks, 6 months, 1 year, 2 years, or more.

An animal reconstituted or repopulated with an expanded population of HSCs as described herein is another aspect of the invention.

As described herein, HSCs produced by the methods of the invention can be used for bone marrow transplantation, *e.g.*, human bone marrow transplantation. Human autologous and allogeneic bone marrow transplantation are currently used as therapies for diseases such as leukemia, lymphoma, and other life-threatening diseases. For these procedures, however, a large amount of donor bone marrow must be removed to ensure that there are enough cells for engraftment. The methods of the present invention alleviate this problem. Methods of transplantation are known to those skilled in the art.

The discovery that cells may be expanded *ex vivo* (*in vitro*) and administered intravenously provides the means for systemic administration. For example, bone marrow-derived stem cells may be isolated with relative ease and the isolated cells can be cultured according to methods of the present invention to increase the number (expand) of HSCs available. Intravenous administration of the expanded HSCs also affords ease, convenience and comfort at higher levels than other modes of administration. In certain applications, systemic administration by intravenous infusion is more effective overall. In another embodiment, the stem cells are administered to an individual by infusion into the superior mesenteric artery or celiac artery. The cells may also be delivered locally by irrigation down the recipient's airway or by direct injection into the mucosa of the intestine.

With respect to cells as administered to a patient, an effective amount may range from as few as several hundred or fewer to as many as several million or more. As used herein, an "effective amount" is that amount which produces the desired result. In specific embodiments, an effective amount may range from about

10^3 to about 10^8 or more. It will be appreciated that the number of cells to be administered will vary depending on the specifics of the disorder to be treated, including but not limited to size or total volume/surface area to be treated, as well as proximity of the site of administration to the location of the region to be treated, among other factors familiar to the medicinal biologist.

The effective amount can be determined by the skilled physician and depends on typical parameters, *e.g.*, the condition to be treated, the duration of treatment, the tolerance of the patient, *etc.* In one embodiment, between about 10^4 and about 10^{12} cells per100 kg person are administered per infusion. In another embodiment, about $1x10^4$ to about $5x10^6$ cells are infused intravenously per 100 kg person. In another embodiment, between about $1x10^6$ and about $5x10^8$ cells are infused intravenously per 100 kg person. For example, dosages such as about $2x10^7$ cells per 100 kg person $4x10^7$ cells per 100 kg person, about $8x10^7$ cells per 100 kg person, about $2x10^8$ cells per 100 kg person, about $4x10^8$ cells per 100 kg person, about $8x10^8$ cells per 100 kg person, about $2x10^9$ cells per 100 kg person, about $4x10^9$ cells per 100 kg person, about $8x10^9$ cells per 100 kg person, about $2x10^{10}$ cells per 100 kg person, about $4x10^{10}$ cells per 100 kg person, and about $8x10^{10}$ cells can be infused per 100 kg person. See, *e.g.*, deLima *et al.*, Bone Marrow Transplant (2008): 41(9):771-8.

In some embodiments, a single administration of cells is provided. In other embodiments, multiple administrations are used. Multiple administrations can be provided over periodic time periods such as an initial treatment regime of 3 to 7 consecutive days, and then repeated at other times.

Expanded HSCs can be administered for as necessary for an effective period. The terms "effective period" (or time) and effective conditions refer to a period of time or other controllable conditions (*e.g.*, temperature, humidity for *in vitro* and *ex vivo* methods), necessary or preferred for an agent or pharmaceutical composition to achieve its intended result.

The expanded HSCs as produced by the methods described herein can be administered with a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" (or medium), which may be used interchangeably with the term "biologically compatible carrier" or "biologically

compatible medium", refers to reagents, cells, 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 complication commensurate with a reasonable benefit/risk ratio. As described in greater detail herein, pharmaceutically acceptable carriers suitable for use in the present invention include liquids, semi-solid (*e.g.*, gels) and solid materials (*e.g.*, cell scaffolds). As used herein, the term biodegradable describes the ability of a material to be broken down (*e.g.*, degraded, eroded, dissolved) *in vivo*. The term includes degradation *in vivo* with or without elimination (*e.g.*, by resorption) from the body. The semi-solid and solid materials may be designed to resist degradation within the body (non-biodegradable) or they may be designed to degrade within the body (biodegradable, bioerodable). A biodegradable material may further be bioresorbable or bioabsorbable, *e.g.*, it may be dissolved and absorbed into bodily fluids (water-soluble implants are one example), or degraded and ultimately eliminated from the body, either by conversion into other materials or by breakdown and elimination through natural pathways.

Several terms are used herein with respect to transplantation therapies, also known as cell-based therapies or cell replacement therapy. The terms autologous transfer, autologous transplantation, autograft and the like refer to treatments wherein the cell donor is also the recipient of the cell replacement therapy. The terms allogeneic transfer, allogeneic transplantation, allograft and the like refer to treatments wherein the cell donor is of the same species as the recipient of the cell replacement therapy, but is not the same individual. A cell transfer in which the donor's cells have been histocompatibly matched with a recipient is sometimes referred to as a syngeneic transfer. The terms xenogeneic transfer, xenogeneic transplantation, xenograft and the like refer to treatments wherein the cell donor is of a different species than the recipient of the cell replacement therapy.

The expanded HSCs provided by the methods described herein are also useful to produce cells of specific hematopoietic lineages. The maturation, proliferation and differentiation of expanded hematopoietic cells into one or more selected lineages may be achieved by culturing the cells with appropriate factors

including, but not limited to, erythropoietin (EPO), colony stimulating factors, *e.g.*, GM-CSF, G-CSF, or M-CSF, SCF, interleukins, *e.g.*, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-13, *etc.*, or with stromal cells or other cells which secrete factors responsible for stem cell regeneration, commitment, and differentiation. Thus, methods of the invention can further comprise culturing the HSCs with appropriate factors to produce specific hematopoietic lineages or cells.

Additionally, the expanded HSCs and/or cells of specific lineages produced from expanded HSCs are suitable for use in *ex vivo* gene therapy. For example, in *ex vivo* gene therapy, cells are removed from a patient, and while being cultured in vitro are genetically modified. Generally, a functional replacement gene is introduced into the cells *via* an appropriate gene delivery vehicle/method (transfection, infection, transduction, homologous recombination, *etc.*) using an expression system as needed. The genetically modified cells are expanded in culture and returned to the patient. These genetically re-implanted cells will express the transfected genetic material in situ in the patient. Thus, in one embodiment is expanded HSCs and/or cells of specific lineages produced from expanded HSCs, that are genetically modified cells.

Methods for genetically modifying cells (*e.g.,* MSCs and/or HSCs) with a transgene are known in the art. Typically, a nucleic acid molecule comprising a nucleic acid sequence of interest is introduced into the MSC or HSC, or cell derived from the MSCs or HSCs, in a form suitable for expression of the nucleic acid sequence of interest in the cell. As appropriate, the nucleic acid molecule can include coding and regulatory sequences for transcription of a gene (or portion thereof) and, when the gene product is a protein or peptide, translation of the gene acid molecule include promoters, enhancers and polyadenylation signals, as well as sequences necessary for transport of an encoded protein or peptide, for example N-terminal signal sequences for transport of proteins or peptides to the surface of the cell or secretion.

The methods described herein are *ex vivo* methods, although long-term culture (*e.g.*, longer than one, two, three or more days) of cells is sometimes referred to as an "in vitro" method. As understood by the person of skill in the art, "ex vivo" refers to experimentation or measurements done in or on living tissue or cells in an

artificial environment outside the organism. HSCs isolated (*e.g.*, from bone marrow or cord blood) and cultured as described herein is considered "*ex vivo*" experimentation, but is also sometimes referred to as "*in vitro*" since culture takes place under artificial conditions *in vitro*.

The foregoing will be apparent from the following more particular description of example embodiments of the invention, and as illustrated in the accompanying drawings. The following Examples are not intended to limit the present invention in any way.

EXAMPLES

EXAMPLE 1

Methods

MSCs obtained from the bone marrow of a human donor were expanded in a MSCBM culture media supplemented with growth factors according to manufacturer's instructions (Lonza, Switzerland). Bone marrow MSCs (BM-MSCs) were engineered to express the human Angptl5 after transduction with a lentiviral vector (PBL2) at a MOI of 5. The Homo sapiens angiopoietin-like 5 (Angptl5) (NCBI accession number NM_178127.2), prepared as transfection-ready DNA (Origene, Rockville, USA) was cut from the pCMV6-XL5 vector using the restriction enzyme Not1, and then cloned into the PLB2 lentiviral vector under the EF1a promoter which drives gene expression of both the inserted nucleotide sequence and green fluorescent protein (GFP). MSCs expressing GFP were engineered to express GFP after transduction with a lentiviral vector at a MOI of 5. After 3-4 days post transduction, MSCs were counted and plated in a 24-well plate at 5 x 10^5/well.

HSCs obtained either from a commercial source (Allcells, LLC, Calif. USA) or purified in our facility using magnetic beads targeting (selecting for) both CD34 and CD133 (StemCell Technologies) were cultured over the MSC layer at a ratio of 1:5 (HSC:MSC ratio) i.e., 10^5 HSC cells and 5x10^5 MSC cells/well in a 24 well plate. The MSC were pre-plated overnight before the addition of HSCs in a serum free-media (StemSpan®, StemCell Technologies) containing IGFBP2, SCF, TPO, FGF-1 and heparin. Fresh media was added every 2 days starting at Day 3.

In vitro expansion of HSCs co-cultured with MSCA5.

Interactions between MSC and primitive HSCs (as used herein, "primitive" HSCs refers to non-expanded HSCs) contained in the cord blood sample were observed by microscopy (FIG. 1). Cord blood HSCs were obtained as described above, either from a commercial source (Allcells, LLC, Calif. USA) or purified in our facility using magnetic beads targeting (selecting for) both CD34 and CD133 (StemCell Technologies). HSCs started dividing at Day 3 and by Day 11 the expanded cells covered the entire MSC layer. Most of the HSCs were strongly attached on the MSC layer, since around 10 % of HSCs remained attached to the MSCs after several serial washes with PBS, and they were able to re-expand when fresh medium was added. Cultured HSCs can also be transferred to freshly plated MSCs at a ratio of 1:5 (HSC:MSC) for long-term expansion, as described herein.

Visual evidence of cellular interaction and expansion between HSCs and MSC.

Co-culture of unmanipulated (freshly isolated) HSCs with MSC-GFP (also referred to herein as MSCGFP) or MSCA5 (also referred to herein as MSC-Angptl5) were visualized by microscopy during different time points of the expansion. At Day 3, a confluent monolayer of MSCs fills the field. By Day 11, this layer is shadowed by the massive proliferation of hematopoietic cells. (FIG. 1, × 10 magnification).

Cultured cells were analyzed for the expression of stem cell markers CD34 and CD133 and compared to cells cultured in cell free conditions (CFC). HSCs were separated from MSCs by mechanical means (*e.g.*, pipetting) before analysis by flow cytometry, using standard techniques. At Day 11, HSCs cultured on MSCA5 showed a 220 fold increase (FI) of total nuclear cells (TNC) and a 64 FI of double positive cells (DPC) (CD34+CD133+). HSCs cultured on MSC-GFP lacking the expression of Angptl5 or in cell free cultured media containing Angptl5 but no MSCs showed a lower expansion (110 and 100 FI of TLC, 28 and 20 FI of DPC respectively) (FIGS. 2A-2B).

The percentage of DPC starting at 96% decreased through the expansion in all culture conditions and reached 30, 26, and 19% at Day 11, for MSCA5, MSCGFP ("MSC-CT") and CFC ("Cell-free CT" or "Cell-free media") respectively (FIGS. 3A-3C).

At Day 11, HSCs were re-cultured by mechanically separating (pipetting) the HSCs from the original culture of MSCs and re-seeding the separated HSCs on a fresh layer of MSCs at the initial ratio of 1:5 (HSC:MSC). HSCs cultured on MSCs were able to expand further, and the FI of MSCA5 and MSC-GFP reached 153 and 110x, respectively. HSCs cultured under CFC were unable to extend further and most of the cells stop dividing and significant numbers of dead cells were detected (FIGS. 2A 2B and 3A, 3B).

Cell-to-cell contact is necessary for HSCs ex vivo proliferation (FIGS. 4A and 4B)

To determine whether the MSC-secreted factors (*e.g.*, Angptl5 and other naturally secreted factors) are sufficient along with the supplemented factors (IGFBP2, SCF, TPO, FGF-1 and heparin) to obtain the high proliferation rate observed in the co-culture system, HSCs were cultured in transwells (TW). The need for Angptl5 was tested by supplementing the MSCGFP co-culture condition with recombinant Angptl5 (rAngptl5). Co-culture of HSCs with MSCA5 or with MSCGFP plus recombinant Angptl5 resulted in a similarly high FI of the DPC (48 and 43x, respectively).

Direct cell-to-cell contact is necessary for HSC proliferation. Cells were cultured in transwells such that the HSCs and MSCA5 cells were cultured in the same well, but were separated from each other by a physical barrier, thereby removing cell-to-cell contact between HSCs and MSCs. HSCs cultured in this manner showed lower levels of HSC expansion and were comparable to CFC (7 and 7.5x, respectively). These results provide direct evidence of synergistic effect of cell-to-cell contact and Angptl5 in promoting *ex vivo* HSC expansion.

Relevance of the different growth factors supplementing the co-culture system.

The defined serum-free medium that was used in all co-culture conditions contains three well-characterized hematopoietic growth factors, SCF, TPO, and FGF-1, together with two recently identified novel hematopoietic growth factors: Angptl5, and IGFBP2. To study the importance of each molecule on the expansion of HSCs, the different factors were subtracted from the culture conditions of HSC cells cultured on the MSCs feeding layer expressing either GFP or Angptl5. Although the percentage of DPCs was not affected in the different subtracted conditions (around 30%), the effect on the proliferation was considerable. In the

initial condition where HSC were cultured in the plain serum-free media without any supplement, this resulted in a complete failure to expand and to a 70% of cell death by Day 11 of the experiment. Subtracting FGF, IGFPBP2, or both, resulted in a 40, 60 and 80%, respectively, decrease in DPC fold increase (FIG. 5). This indicates that MSCs alone cannot support the *in vitro* proliferation of HSCs. The MSCs require themselves growth factors such as FGF to be able to express secreted molecules implicated in the feeding role they are playing. (Wagner *et al.*, Stem Cells. 2007: 25(10):2638-47; and Zhang, Tissue Eng. 2006: 12(8):2161-70).

Moreover, IGFBP2 seems to act synergistically with the secreted Angptl5 as the absence of each or both factors resulted in similar decrease of cell proliferation. Thus, the removal of these growth factors either individually or in combination failed to produce the same proliferation rate as the combination of IGFBP2 and Angptl5.

Long term ex vivo expansion of HSCs.

In the co-culture system established above, a decrease of the percentage of DPCs was observed after 14 days, from 60% at Day 6 to about 12% at Day 18. This was associated with a slowing of the absolute cell proliferation. Cells were cultured for several weeks and monitored for both the proliferation and percentage of DPCs. However, cells cultured without any feeding layer completely stopped their division and went through apoptosis. Around Day 30, HSCs cultured on MSCA5 and MSCGFP showed a new rise of the DPC population to reach a percentage of 30% and 45% at Day 40. Although the proliferation rate was slower than the beginning, the accumulated FI was 12,000 and 2,600 respectively (FIG.6). This result indicates a significant role of the MSC, in providing microenvironment capable of supporting a long term expansion of HSCs.

Ability of expanded HSCs to reconstitute immunocompromised mice.

Two major human stem/progenitor cell populations have been described using immunodeficient mice engrafted with human HSC: one is termed short-term repopulating cell (STRC), the other long term repopulating cell (LTRC).

Short-Term Repopulating Cell Engraftment.

After 10 days co-culture of HSCs (Allcells, CAlif. USA) with MSCA5 cells, 10^5 human stem/progenitor cells (CD34+CD133+) were transplanted into

sublethally irradiated NOD/SCID gamma chain newborn mice (Jackson Labs). Human cell engraftment in the blood of the recipients was determined 8 weeks later. Mice injected with cells cultured with or without the presence of feeder cells showed a similar rate of human CD45 in the blood cells (4-6%). This percentage reached higher levels at 12 weeks post-engraftment (30-60%). At 14 weeks post-transplantation, mice were euthanized and assessed for the presence of human cells in the bone marrow and different lymphoid organs (FIGS. 7B and 7C). A striking difference observed when comparing both groups was the presence of large number of CD3+ cells in blood, spleen (FIG. 7B, C) and other organs of mice injected with HSC from MSCA5 culture. Also noted was an increase of myeloid progenitors (CD33+) and mature cells (CD14+).

Additional studies were performed using hematopoietic stem/progenitor cells that were cultured on the MSCA5 layer for 40 days and then injected into mice. HSCs cultured for a longer time on MSCs layer expressing Angptl5 do not lose their homing and reconstitution abilities as they show similar engraftment percentage as short-term cultured cells (FIG. 8A). Moreover , they present similar percentage of CD3, CD33, CD66b, CD14 positive cells when compared to cells that were cultured in same condition for shorter time (11 days).

EXAMPLE 2

Specific Studies: Optimizing protocols for isolating the rare HSCs from unmobilized peripheral blood of adult donors with high purity and yield.

HSCs are a rare population in unmobilized peripheral blood. Existing methods are not sufficient to purify these extremely rare cells without significant losses. Therefore, an efficient purification method has been developed as described herein.

The frequency of CD34+CD133+ HSCs in adult peripheral blood is ~0.05% after removal of erythrocytes and granulocytes (see FIG. 9B). Described herein is a one-step magnetic enrichment procedure for isolating HSCs from adult peripheral blood. In this procedure, red blood cells in buffy coat are rosetted with granulocytes (*e.g.*, using RosetteSep® (Stem Cell Technologies, Vancouver, BC) performed in accordance with manufacturer's instructions) and removed by Ficoll centrifugation (for example, using Leucosep™ (Greiner Bio-One GmbH, Germany) in accordance

with manufacturer's instructions). The resulting peripheral blood mononuclear cells (PMBC) are incubated with magnetic nanoparticle-conjugated anti-CD34 antibody in the presence of Fc blocker. After washing, anti-CD34 stained cells are magnetically recovered. By this one-step procedure, at least, or about 34% of the isolated cells were CD34+CD133+ HSCs.

In an alternative purification protocol, PBMC can be labeled with anti-CD34 and anti-CD133 antibodies simultaneously. Both antibodies have been used simultaneously to isolate HSCs from cord blood with near 100% purity (CD34+CD133+) and 100% yield. It is expected that simultaneous use of both anti-CD34 and anti-CD133 will also increase the purity and yield of HSCs isolated from adult peripheral blood. For *in vitro* expansion, the purity of HSCs is preferably >80% CD34+CD133+, although low percentages also work (see below). In a further alternative method, purification procedures can be repeated to yield HSCs with ~80% purity or more.

Specific Studies: Optimizing protocols for expanding adult HSCs in vitro to obtain large numbers of HSCs.

Human HSCs from adult peripheral blood have not been successfully expanded *in vitro* using other methods. An efficient method has to be developed to expand the numbers of HSCs in order to reconstitute sufficient numbers of mice for subsequent study.

Initial tests were conducted to determine whether HSCs from adult peripheral blood can be expanded in the cell-free medium and the co-culture system as described herein. In the initial trial, HSCs (~50% CD34+CD133+) isolated from unmobilized adult peripheral blood were cultured in cell-free medium, StemSpan® medium (Stem Cell Technologies, Vancouver, BC) was supplemented with 10 ng/ml SCF, 20 ng/ml TPO ,10 ng/ml FGF-1, 100 ng/ml IGFBP2 ,500 ng/ml Angplt5, or on a monolayer of bone marrow-derived MSC that express lentivirally transduced A5 (BM-MSC-A5), or on a monolayer of embryonic stem cell-derived MSC that express lentivirally transduced A5 (ES-MSC-A5). As a positive control, HSCs (>95%CD34+CD133+) from cord blood were cultured under the same three conditions.

After 11 days, cells in the cultures were counted and analyzed for CD34 and CD133 expression to estimate HSCs expansion. Cord blood HSCs expanded ~30 fold in cell-free medium and 60-70 fold in MSC co-cultures (see FIG. 9A). Although HSCs from adult peripheral blood did not expand in the cell-free medium, they expanded ~10 fold in BM-MSC-A5 co-culture and ~20 fold in ES-MSC-A5 co-culture. Thus, adult HSCs can be expanded in the co-culture, especially with ES cell-derived MSC.

Further protocols for expanding adult HSCs include the following three approaches. First, in the method described above, the starting HSCs were ~50% CD34+CD133+. HSCs with higher purity can be used instead (see above protocols described to optimize isolating rare HSCs from unmobilized peripheral blood from adult donors), for example, at least, or about 60%, at least, or about 70%, at least, or about 80%, or greater HSC purity. This is expected to reduce competition from non-HSCs in the co-culture. Second, the lack of HSC expansion in cell-free medium suggests that adult HSCs require different growth factors than HSCs from cord blood or fetal liver. Thus, expansion of adult HSCs in cell-free medium can be optimized with the addition of further angiopoietins to promote adult HSCs expansion in the cell free medium. Alternatively, adult HSCs can be co-cultured with MSCs expressing additional angiopoietins. Third, recent studies suggest that three-dimensional biomaterial-based microcarriers and dynamic culture conditions enhances the expansion of HSCs. Thus, in one embodiment, HSCs, such as adult HSCs can be cultured using a three-dimensional material, such as microcarriers. The use of a three-dimensional culture system can be used with or without co-culturing MSCs.

One unit of blood (450ml) can yield ~1 x 10^9 PBMC. Based on ~0.05% of CD34+CD133+ HSCs in PBMC, there are 5 x 10^5 HSCs in one unit of blood. Assuming a conservative HSC recovery of 20-40% during purification, 1-2 x 10^5 HSCs can be obtained from one unit of blood. Based on expansion of HSCs by 20 fold and reconstitution with 100,000 HSCs per recipient (*e.g.*, a mouse model such as immunodeficient (NOD/scid, $\gamma c^{-/-}$ (NSG)) mice), sufficient numbers of HSCs should be obtainable from one unit of blood to reconstitute a cohort of 20-40 mice.

EXAMPLE 3

Experimental Procedures:

Cell purification: Purified human $CD133^+$ cord blood cells were purchased from AllCells (California). Alternatively, umbilical cord blood was obtained from the National Disease Research Interchange (NDRI) or the Singapore Cord Blood Bank. Red blood cells (RBCs) were removed by Ficoll-Hypaque density gradient. $CD34^+$ cells were purified with the RosetteSep system using the CD34 positive selection kit (Stem Cell Technologies, Vancouver, BC). The purity of purified cells was 90 to 99% $CD34^+$. On average, 95% of the purified $CD34^+$ cord blood cells were also $CD133^+$. Following *in vitro* expansion (see below), $CD133^+$ cells were purified by staining cells with PE-conjugated anti-CD133 (E-Biosciences) followed with a PE positive selection kit (Stem Cell Technologies, Vancouver, BC). The purity of purified cells was 85 to 95% $CD133^+$, almost of which were $CD34^+$.

Feeder cell-free (FCF) culture of $CD34^+CD133^+$ cord blood cells: Cryopreserved or freshly isolated $CD34^+CD133^+$ cord blood cells were cultured in vitro as described (Zhang *et al.*, 2008, Blood *111*, 3415-3423). Briefly, StemSpan® medium (Stem Cell Technologies, Vancouver, BC) was supplemented with 10 ng/ml SCF (R&D Systems, Minneapolis, MN), 20 ng/ml TPO (R&D Systems, Minneapolis, MN), 10 ng/ml FGF-1 (Gibco), 100 ng/ml IGFBP2 (R&D Systems, Minneapolis, MN), 500 ng/ml Angplt5 (Abnova, Taiwan), 500 μg/ml of heparin (Sigma), 1x penicillin and streptomycin (Gibco) to obtain the expansion media. About 10^4 CD34+CD133+ cord blood cells were plated in a 96 well round bottomed plate, in 200 μl/well of the expansion media. Cells were transferred to a 6-well plate 4 days later and fresh media was added to keep cells at 200,000/ml one day after the transfer. Cells were supplemented with more fresh media 2-3 days later, in order to keep them at 700,000 cells/ml and then allowed to expand until the end of the 11-day culture. For the FCF culture with activin A, freshly isolated CD34+ CD133+ cord blood cells were plated in 96-well plate at 10^4 cells/well and cultured in the expansion media with or without addition of 50ng/mL of recombinant human activin A (PeproTech, Rocky Hill, NJ). Cells were transferred to a 24-well plate 3 days later and fresh media (with or without activin A) was added every two days to maintain cell concentration at approximately $5x10^5$ cells/mL.

Angplt5-epressing MSCs: The human Angptl5 (DNA NM_178127.2) was excised from the pCMV6-XL5 vector (Origene, Rockville, USA) using Not1, and cloned into the pLB2 lentiviral vector at Not I site. The resulting vector encodes both Angplt5 and GFP under the same EF1a promoter (provided by Dr. Patrick Stern of Massachusetts Institute of Technology). To produce lentiviruses, 293FT cells were co-transfected with lentiviral vectors, the HIV-1 packaging vector Delta8.9 and the VSVG vector. Supernatants containing lentiviruses were harvested on day 2 or 3 and concentrated by ultracentrifugation at 25,000g for 90 min and frozen until use. Lentivirus titers were 10^7-10^8/mL based on FGP expression on 293FT cells.

Human MSCs from the bone marrow of adult donors were purchased commercially (Lonza, Basel, Switzerland, or Stem Cell Technologies, Vancouver, BC). MSCs were cultured in MesenCult MSC Basal Medium and Mesenchymal Stem Cell Stimulatory Supplements (Stem Cell Technologies, Vancouver, BC) at a density of 5,000-6,000 cells per cm^2 in a T175 flask and passaged prior to reaching 70% confluency. MSCs from 2nd to 4th passages were transduced with a lentiviral vector (pLB2) expressing GFP alone or both FGP and Angplt5 at a MOI of 5. Four to five days post transduction, 30-45% of cells were GFP+ by flow cytometry analysis. The mixture of transduced and non-transduced MSCs were expanded and used for co-cultures.

Co-culture of $CD34^+CD133^+$ cord blood cells: MSC-A5 or MSC-GFP were plated in a 24-well plate at 5 x 10^4 cells/well overnight. $CD34^+CD133^+$ cord blood cells were added to the culture at a cord blood cell to MSC ratio of 1:5 (i.e., 10^4 cord blood cells for $5x10^4$ of pre-plated MSCs per well). The expansion media was used for co-culture except for the addition of Angptl5. The initial volume of culture media was adjusted to 300µl/well, and fresh media was added every 2 days starting on the 3rd day of culture. At different time points of the culture, hematopoietic cells were resuspended by carefully pipetting 5-7 times (avoiding the detachment of the MSCs), counted and analyzed by flow cytometry to obtain the total cell number and the number of DP cells. After 11 or 14 days of culture, hematopoietic cells were resuspended thoroughly (to loosen strongly attached cells to the feeder layer), analyzed for CD34 and CD133 expression. For co-cultures with follistatin, the

culture was done the same as above except 200ng/mL of the recombinant human follistatin (PeproTech) was added into some wells.

Mice and intracardiac injection: NSG mice were obtained from the Jackson Laboratory and bred in the animal facilities at Massachusetts Institute of Technology, Nanyang Technological University, and National University of Singapore. Pups within the 48 hrs of birth were sublethally irradiated with 100 rads using a Cesium source and engrafted with either expanded or unexpanded cells by intracardiac injection. For engraftments, either 10^5 purified $CD34^+CD133^+$ cord blood or expanded cells in 50 µl were injected per recipient, or total expanded cell suspension containing 10^5 $CD34^+CD133^+$ cells in 50 µl were injected per recipient. For limiting dilution assays, a non-toxic green food dye (Apple Green Liquid Dye [containing tartrazine E142], Bake King, Gim Hin Lee (Pte) Ltd, Singapore) was mixed with the progeny of various number of starting cells just before injection to monitor the success of the injection. All research with human samples and mice was performed in compliance with the institutional guidelines.

Serial and competitive reconstitution assays: Serial reconstitution was performed as follows: Fourteen weeks after the reconstitution, bone marrow cells were harvested from both femurs and tibia of primary recipients. Human $CD34^+$ cells were stained with PE-conjugated anti-CD34 followed with a PE positive selection kit (Stem Cell Technologies, Vancouver, BC). $CD34^+$ cells were pooled from different mice reconstituted with expanded cells from the same cord blood donor and then 10^4 were injected into sublethally irradiated newborn pups. For the competitive reconstitution assay, $CD34^+CD133^+$ cord blood cells were isolated from a HLA-$A2^+$ and a HLA-$A2^-$ cord blood donor. A portion of the cells from each donor was expanded in MSC-A5 co-culture for 11 days and $CD34^+CD133^+$ cells purified. The expanded and unexpanded cells from HLA-$A2^+$ donor were mixed in equal number with unexpanded or expanded cells from HLA-A^- donor and injected into sublethally irradiated newborn pups.

Analysis of reconstituted mice: At the indicated time points after engraftment, blood, spleen and/or bone marrow were harvested for various analyses. For flow cytometry analysis, single cell suspensions were prepared. Cells were counted and stained with antibodies specific for human CD3, CD11c, CD14, CD15,

CD19, CD33, CD34, CD45, CD56, HLA-DR (Biolegend), CD133 (Miltenyi or EBiosciences), and Murine CD45.1 (Biolegend). Stained cells were analyzed on FACScalibur, FACS-Canto or LSR II cytometers (Beckton Dickinson). Dead cells were excluded from analysis by DAPI staining. Analyses were performed with FlowJo Software (Tree-Star). SRC frequency in limiting dilution assays was determined by the method of maximum likelihood with L-CALC software (StemCell Technologies).

Hematoxylin and Eosin (H&E) staining was performed on formalin-fixed, frozen spleen sections. Immunofluorescence staining was performed on 75% acetone/25% ethanol fixed, frozen spleen sections. Monoclonal antibody to CD20 (clone L26, Abcam, UK) and polyclonal antibodies to CD3 (Abcam) were used as primary antibodies. Alexa fluro647 donkey anti-mouse IgG (Invitrogen, USA) and Alexa fluro546 donkey anti-rabbit IgG (Invitrogen) were used as secondary antibodies. H&E stains were visualized with a light microscope and immunofluorescence stains were visualized using a slide scanner (Mirax Midi, Zeiss).

Immunization and ELISPOT assay: Sixteen weeks after engraftment, mice were immunized intraperitoneally (i.p.) with 10 µl of tetanus toxoid vaccine adsorbed on hydrated aluminium hydroxide (Tetavax, Sanofi Pasteur, France) diluted in 90 µl PBS, representing 1/50 of the recommended vaccination dose for a human adult. Mice were boosted twice with the same dose in three-week intervals. Two weeks after the third immunization, mice were sacrificed and the frequencies of IFN-g secreting cells in the spleen were measured by ELISOPT assay (EBiosciences, San Diego, CA). Briefly, single cell suspensions were prepared from spleens, counted and analyzed by flow cytometry for the frequency of human $CD3^+$ T cells. $5x10^5$ $CD3^+$ cells were plated per well in a 96-well flat-bottomed plate (Multiscreen-IP, Millipore, MA, USA) that was coated overnight at 4°C with anti-IFN-g monoclonal antibody. For T cell stimulation, 10ng/ml of PMA or 0.5 µg/ml of the tetanus toxin peptide (830-843) (Genscript, USA) were added to the culture. After 48 hours of incubation at 37°C, 5% CO2, IFN-g immunospots were detected according to the manufacturer's instructions. The spot were counted using an

ImmunoSpot S5 Versa Analyzer (Cellular Technology Ltd. Ohio, USA) and analyzed with ImmunoCapture software (Analysis Software).

Results

Combination of co-culture and selected growth factors enhance expansion of $CD34^+CD133^+$ cord blood cells. To investigate whether MSCs and recently identified growth factors, such as IGFBP2 and Angptl5, synergize to enhance HSC expansion, $CD34^+CD133^+$ double positive cells (DPCs) were cultured on an MSC feeder layer in the presence of additional growth factors described by Zhang *et al.*, 2008, Blood *111*, 3415-3423. Human Angptl5 was required at very high concentrations in this culture system requiring massive bolus doses. As described herein, Applicants discovered that constant production of Angptl5 by the feeder cells is an advantageous method. Therefore, primary human bone marrow MSCs were transduced to express Angptl5 using a lentiviral vector (MSC-A5). A control population of MSCs were transduced with a GFP control vector (MSC-GFP). Magnetically purified $CD133^+$ cord blood cells, which were also $CD34^+$, were cultured with a confluent MSC-A5 or MSC-GFP feeder layer at a starting ratio of 1:5. All cultures included SCF, FGF, IGFBP2, and TPO in the culture media as described (Zhang *et al.*, 2008, Blood *111*, 3415-3423). For comparison, purified $CD133^+$ cells were also cultured in FCF culture, i.e., serum-free medium supplemented with Angptl5, SCF, FGF, IGFBP2, and TPO. At the start of the co-culture, hematopoietic cells were observed to attach to MSC feeder layer (FIG. 10A). By day 5, the density of hematopoietic cells was noticeably increased. By day 7, proliferation of hematopoietic cells was evidenced by cell density and cell counting (FIGS. 10A, 10D). By day 11, hematopoietic cells had completely covered the MSC-A5 feeder layer (FIG. 10A).

To monitor HSC expansion, cultured cells were assayed for CD34 and CD133 expression because SRCs reside in the $CD34^+CD133^+$ DP fraction and their expansion in culture correlates with DPC growth. At the beginning of the co-culture, more than 95% of the input cells were $CD34^+$ and $CD133^+$. After culture for 7 days, the percentage of DP cells decreased to ~50% in MSC-A5 or MSC-GFP co-culture and ~30% in FCF culture (FIGS. 10B, 10C). The percentages continued to decrease and by day 14, they were ~15% in co-cultures and ~10% in FCF culture. Despite

the decrease of the percentages of DP cells in the cultures, because the total cell numbers increased dramatically (FIG. 10D), the actual numbers of DP cells were increased ~60 fold in MSC-A5 co-culture, ~29 fold in MSC-GFP co-culture, and ~20 fold FCF culture at day 11 (FIG. 10E). By day 14, while the number of DP cells did not increase significantly in the FCF culture, the number of DP cells increased to ~150 and ~60 folds in MSC-A5 and MSC-GFP co-cultures, respectively. When $CD34^+$ cells isolated from fetal liver and mobilized peripheral blood were cultured using the same conditions, expansion of DP cells in MSC-A5 co-culture was also significantly higher than that in the FCF culture. These results show that combination of MSCs and the different growth factors can support a much more robust $CD34^+$ $CD133^+$ HSC expansion.

Both cell-to-cell contact and soluble factors contribute to the enhanced expansion of $CD34^+CD133^+$ cord blood cells. To investigate the role of cell-to-cell contact in the expansion of DPCs, co-cultures using transwells to prevent direct contact between MSC-A5 and $CD34^+CD133^+$ cord blood cells were performed. During 11 days of culture, both the total numbers of hematopoietic cells and the numbers of DP cells were similar in the transwell co-culture and the FCF culture, however the number of cells in the co-cultures where cord blood cells were free to contact MSCs were much higher (FIGS. 4A, 4B). Co-culture with MSC-GFP led to greater expansion of both cells and DPCs and co-culture with MSC-A5 lead to the greatest expansion of cells and DPCs (FIGS. 4A, 4B). Addition of recombinant Angplt5 to MSC-GFP co-culture resulted in enhancement of both cell and DPC expansion to similar levels to those seen in MSC-A5 co-culture. These results suggest that contact between MSCs and $CD34^+CD133^+$ cord blood cells are a significant factor contributing to the enhanced expansion of DPCs. They also confirm that Angplt5 further enhances expansion and that while use of MSC-A5 reduces the cost of culture there is no additional improvement to DPC expansion from producing Angptl5 *in situ*.

While the entire cocktail of cytokines used by Zhang *et al.*, 2008, Blood *111*, 3415-3423, is optimal for feeder cell free culture, one or more of these factors may be unnecessary in co-culture. To test whether this is the case co-cultures without adding specific growth factors were tested. For ease of comparison, DPC expansion

in culture was normalized to the fully supplemented MSC-A5 co-culture condition. In no condition where growth factors were omitted was DPC expansion equivalent to that seen in the fully supplemented MSC-A5 culture (FIG. 5). When DPCs $CD34^+CD133^+$ cord blood cells were co-cultured with MSC-A5 without the addition of any of the growth factors, they did not expand at all. Addition of just SCF and TPO, which are well established HSC growth factors and broadly used together (Liu *et al.*, 1999, one Marrow Transplant *24*, 247-252; Ohmizono *et al.*, 1997, Leukemia *11*, 524-530), into the above co-culture led to expansion of DP cells, reaching 19% of that obtained under the standard condition. With further addition of FGF or IGFBP2, the relative expansions were 36% and 61%, respectively. Without exogenously added Angplt5 (co-culture with MSC-GFP in the presence of SCF, TPO, FGF, and IGFBP2), the relative expansion was 43% (FIGS. 4A, 4B). These results suggest that all five growth factors contribute to the expansion of DPCs.

Expanded CD34+CD133+ cells give rise to multiple lineages of blood cells in NSG mice. The ability of expanded cells from the co-cultures to reconstitute different lineages of human blood cells in NSG mice was tested. $CD34^+CD133^+$ cord blood cells were either expanded in the MSC-A5 co-culture or the FCF culture for 11 days. Total expanded cells, containing 10^5 DPCs, were engrafted into sublethally irradiated NSG neonates. Fourteen weeks later, the presence of different human blood lineage cells in the peripheral blood, spleen and bone marrow (BM) were analyzed by flow cytometry. Total chimerism (CD45+ cells) in the BM spleen and peripheral blood were similar in both FCF culture and co-culture. Among various human blood lineages cells analyzed, T cells ($CD3^+$), B-lineage cells ($CD19^+$ or $CD19^+IgM^+$), macrophages ($CD14^+CD33^+$), myeloid precursor cells ($CD33^+$), hematopoietic stem/precursor cells ($CD34^+$ or $CD34^+CD133^+$), dendritic cells ($CD11c^+$ $HLA\text{-}DR^+$), and natural killer cells ($CD56^+$) were detected in the blood, spleen or bone marrow (FIG. 11A). In mice reconstituted with co-culture DPCs a significant increase in CD3+ T cells was observed in blood and spleen ($p<0.05$). The reconstitution of all other lineages was similar (see FIG. 16). The significantly higher percentages of $CD3^+$ T cells were already detectable in the blood 8 and 12 weeks after reconstitution (FIG. 11B).

Hemoxylin and eosin (H&E) staining revealed a restoration of the splenic architecture with the appearance of the white pulp in mice reconstituted with co-cultured cells but not in unreconstituted NSG mice (FIGS. 11C, 11D). Furthermore, T cells and B cells were found in the same follicles, with $CD3^+$ T cells residing predominantly in the inner region of the follicles whereas $CD20^+$ B cells residing in the outer region of the follicle (FIGS. 11E, 11F). These results demonstrate that expanded cells from the co-cultures are capable of differentiating into multiple lineages of blood cells in NSG mice and suggest that there is at least partial recovery of secondary lymphoid structure. In addition, the expanded cells from MSC-A5 co-culture supports a significantly higher level of T cell reconstitution in recipient mice than cells expanded in FCF culture.

Expanded cells are capable of stable long-term and efficient secondary reconstitution. The expanded cells were tested for their ability to give rise to stable long-term reconstitution in NSG recipients. Following 11-day expansion in either MSC-A5 co-culture or FCF culture, expanded cells were transferred into sublethally irradiated newborn NSG pups. By 12 weeks following engraftment, the percentages of human leukocytes in the blood were ~50% (FIG. 12A). These percentages increased slightly or were maintained at 24 weeks after engraftment (FIGS. 12A, 12B). No difference in reconstitution was observed between expanded cells from MSC-A5 co-culture or FCF culture in the blood and spleen (FIG. 12A). As shown above, the level of T cell reconstitution in both blood and spleen was significantly higher with expanded cells from co-culture than FCF culture. These results suggest that expanded cells from both protocols possess similar levels of long-term reconstitution activity.

The presence of long-term HSC activity in the expanded cell population was also assessed by serial transfer. Expanded cells from day 11 MSC-A5 co-culture were transferred into irradiated NSG pups. Fourteen weeks after engraftment, human $CD34^+$ cells were magnetically enriched from the bone marrow of primary recipient mice and injected into sublethally irradiated adult NSG mice. Twelve to 16 weeks after, bone marrow cells of the secondary recipient mice were analyzed by flow cytometry for human and mouse $CD45^+$ cells. Among the 14 secondary recipient mice, 11 had 0.1-2% of human $CD45^+$ cells in the bone marrow, one had 11% and

the other two had ~0.03%. A representative mouse is shown in FIG. 12C. These data show that the expanded $CD34^+CD133^+$ cells from MSC-A5 co-culture retain a robust secondary reconstitution potential, suggesting the presence of long-term HSC activity.

Expanded DP cells have the same repopulating capacity as unexpanded cells. Having demonstrated robust long term engraftment of SCID mice by DPCs after culture, the SCID repopulating activity of these cells was assessed by limiting dilution. $CD34^+CD133^+$ cord blood cells were expanded in either MSC-A5 co-culture or FCF culture and purified. Groups of sublethally irradiated pups were engrafted with 5000, 1000, and 400 unexpanded $CD34^+CD133^+$ cord blood cells or the same numbers of purified expanded DP cells (all from the same donor). Successfully injected mice were analyzed for the presence of human leukocytes in the bone marrow 8 weeks later. An arbitrary threshold of 0.05% was selected as the minimum chimerism in the bone marrow for engraftment to have been successful. When 5000 cells were injected 100% of the mice were chimeric, (FIG. 13A). When 1000 and 400 cells were injected the percentages of chimeras decreased, but no significant differences were observed among the three sources of the cells. Furthermore, the percentages of human $CD45^+$ cells in the bone marrow were similar (no statistical difference) irrespective of the source of the DPCs (FIG. 13A). The frequency of SRCs in unexpanded CD34+ CD133+ cells was calculated as 1 in 1024 with 95% confidence interval (CI) of 1/535-1/1958 (FIG. 13B). The frequency of SRCs in MSC-A5 co-culture and FCF culture expanded DP cells was 1 in 12 (with 95% CI 1/6-1/27) and 1 in 28 (with 95% CI 1/13-1/61), respectively. Because DP cells were expanded 58 and 18 fold in MSC-A5 co-culture and FCF culture, respective, SRCs were therefore expanded 85 and 36 fold correspondingly. These results demonstrate that expanded DPCs have the same SCID-repopulating activity as unexpanded cells.

The reconstitution capacity of expanded DPCs cells was also assessed using a competitive repopulation assay. To facilitate the tracking of transferred cells from different donors within the same recipient, two cord blood samples differing in expression of MHC class I gene HLA-A2 were used. DPCs from these samples were mixed in equal numbers for competitive repopulation. To avoid any donor related

bias, the experiment was performed by reciprocally expanding DPCs from both cords and mixing them with unexpanded cells from the other cord. In addition, unexpanded $CD34^+CD133^+$ cells from both cords were mixed and transferred as a baseline control. Eight weeks post-engraftment, bone marrow cells of reconstituted mice were analyzed for human CD45 and HLA-A2. As shown in FIG. 14C, ~50% of human $CD45^+$ cells were $HLA\text{-}A2^+$ and ~50% $HLA\text{-}A2^-$ in all three groups of mice. These results further demonstrate that co-culture expanded SCID repopulation and the degree of expansion correlates with increase in DPC number as previously reported.

MSCs modulate lymphopoiesis through activin A. As shown in FIG. 11B, CD3+ T cell reconstitution was ~2 fold higher in mice engrafted with DP cells from MSC-A5 co-culture than with those from FCF culture. To identify the mechanism by which MSCs stimulate T cell lymphopoiesis, the microarray data of human MSCs isolated from the bone marrow of four healthy donors were analyzed for highly expressed genes with known roles in lymphoid cell development (Djouad *et al.*, 2009, Cloning Stem Cells *11*, 407-416). Activin A, a member of the TGF-ß superfamily, was highly expressed by microarray analysis and further verified by quantitative PCR. Activin A is known to exert pleiotropic effects in numerous biological processes (Shav-Tal and Zipori, 2002, Stem Cells *20*, 493-500), including inhibition of B lymphocyte development *(*Parameswaran *et al., 2008,* Stem Cells Dev *17*, 93-106*; Shoham et al., 2003,* Ann N Y Acad Sci *996*, 245-260*; Zipori and Barda-Saad, 2001,* J Leukoc Biol *69*, 867-873*)*. As both T and B cells are derived from the same lymphoid progenitors, inhibition of B cell development by activin A could lead to an enhanced T cell development. To test this hypothesis, $CD34^+CD133^+$ cord blood cells were cultured in FCF culture in the presence of recombinant activin A. After 11 days of culture, the resulting cells showed minimal differences in cell surface marker. However, 10 weeks after engraftment, recipient NSG mice injected with activin A-cultured cells showed a dramatically higher percentages of $CD3^+$ cells in the peripheral blood when compared to mice injected with expanded cells without the activin A in the culture (FIG. 14A, $82.2 \pm 8.5\%$ vs $39.9 \pm 8.2\%$, $p=3x10^{-6}$). In contrast, the percentages of CD19+ cells were significantly lower in the peripheral blood of mice reconstituted with activin A-

cultured cells as compared to normal FCF cultured cells (13.1 ± 6.6% vs 49.2 ± 7.1%, $p=1.9x10^{-6}$). The differences were also observed when DPCs were purified after culture. Conversely, when follistatin, a natural inhibitor of activin A (de Winter *et al.*, 1996, Mol Cell Endocrinol *116*, 105-114), was added into the MSC-A5 co-culture, the enhanced T cell reconstitution in the recipient mice was completely abolished (FIG. 14C). These results strongly suggest that activin A secreted by MSCs stimulates T cell lymphopoiesis of expanded cells in NSG recipient mice.

Human T cells are functional in the reconstituted mice. The increased T cell development with co-cultured expanded DPCs prompted testing of T cell function. Similarly reconstituted mice with expanded cells from either co-culture or FCF culture were immunized with tetanus toxoid (TT) and boosted twice at an interval of 3 weeks. Two weeks after the last immunization, splenocytes were assayed for IFN-γ expression by ELISPOT assay. Without immunization, significant numbers of IFN-γ immunospot were detected only if splenocytes were stimulated with PMA during the assay, regardless of whether the cells were from mice reconstituted with co-culture or the FCF cultured DPCs (FIG. 15A). Similarly, significant numbers of spot were detected in PMA stimulated splenocytes from immunized mice, regardless of the source of DPCs. As expected, with TT stimulation of splenocytes in the culture, significant numbers of spot were detected only in TT immunized mice. However, the average numbers of spots were significantly more in mice reconstituted with cells from the co-culture than the FCF culture ($p<0.005$). These results suggest that enhanced T cell development leads to an enhanced T cell response in mice reconstituted with co-culture expanded cord blood cells.

Discussion

Described herein is an improved method to expand HSCs from umbilical cord blood by combining the HSC growth factors recently identified by Zhang *et al.*, 2008, Blood *111*, 3415-3423, with a bone marrow-derived MSC feeder layer. The combination of SCF, TPO, FGF, IGFBP2 and Angptl5 synergizes with a primary human BM MSC feeder layer to dramatically increase overall expansion of SCID repopulating cells. An ~70 fold expansion in DPC number and SCID repopulating activity in a short term 11 day co-culture was routinely achieved. The repopulating activity of co-cultured cells was rigorously tested by limiting dilution and in

competition assays. Cultured cells retained long term HSC activity over 6 months *in vivo* and through secondary reconstitution assays. By examining multi-lineage engraftment in NSG mice after co-culture, it was discovered that the MSCs introduce a bias in favor of T cell generation (differentiation), even among purified DPCs. As described herein, Activin A is responsible for this lineage bias. As a result, the resultant T cells are more immunogenic than those from control animals.

Results from the improved co-culture method described herein compare favorably with those reported previously. In the best expansion to date using FCF culture, Delaney *et al.*, 2010, Nat Med *16*, 232-236 reported approximately 180 fold expansion of $CD34^+$ cells following a 17-21 day culture and ~15 fold expansion of SRCs in NSG mice. In a separate study using FCF culture, Suzuki reported 100 fold expansion of $CD34^+$ $CD133^+$ cells in 21 days but only 6 fold expansion of SRCs in mice (Suzuki *et al.*, 2006, Stem Cells *24*, 2456-2465). In the co-culture system described herein, $CD34^+$ (or $CD34^+$ $CD133^+$) cells were expanded ~130 (~60) fold by day 11 and ~480 (~150) fold by day 14 (FIG. 17A, 17B). When assayed in NSG recipients, ~ 70 fold expansion of SRCs was observed by day 11. Using MSC co-culture, 35-50 fold expansion of $CD34^+$ cells was reported previously following 10-13 days culture. The fold of expansion was further enhanced to ~96 fold if MSCs were placed in a 3D configuration using a woven mesh, but only 8-10% of cells remained $CD34^+$(Zhang *et al.*, 2006, Tissue Eng *12*, 2161-2170). However, these previous studies did not rigorously evaluate expansion of SRCs in mice. In one study, injection of progeny of 1500 cells into adult NOD-scid mice yielded 70% chimera as defined by having more than 0.01% of human cells in the bone marrow (Briquet *et al.*, 2010, Haematologica *95*, 47-56). In another study, the progeny of 600 $CD34^+$ cells were injected into each NOD-scid mouse and all five recipients had more than 0.2% human cells in the bone marrow 6 weeks later (Zhang *et al.*, 2006, Tissue Eng *12*, 2161-2170). Among various studies, different strains and age of recipient mice, different numbers and populations (purified or total) of cells from different cords were injected, it is difficult to strictly compare the fold of SRCs. Based on the data available, the co-culture system described herein achieves better expansion of both $CD34^+$ ($CD133^+$) cells and SRCs.

Different from previous co-culture studies, the reconstitution activity of expanded cells from co-culture in long-term, serial and competitive reconstitution has been rigorously tested, as described herein. The expanded cells were able to support stable long-term (at least, or about 6 months) reconstitution. When human $CD34^+$ cells were isolated from the bone marrow of primary recipient mice and transferred into second recipient mice, 12 of 14 recipients had more than 0.1% human cells in the bone marrow 12-16 weeks later. These results suggest that some of the expanded cells still possess the long-term HSC activity. Furthermore, in the reciprocal competitive reconstitution assay using equal number of expanded and unexpanded $CD34^+$ $CD133^+$ cells, the expanded cells were equally as good as unexpanded cells in reconstitution, suggesting that expansion does not significantly diminish SCID repopulating activity of the $CD34^+CD133^+$ cells. These findings further suggest that the co-culture method described herein enhances expansion of HSCs without compromising their reconstitution activity.

MSCs have been shown to stimulate differentiation of HSCs. With the recent development of the NSG mouse model that supports robust lymphoid engraftment, the ability of the co-culture expanded cells for T and B cell reconstitution was examined. While unexpanded or expanded $CD34^+CD133^+$ cells from the FCF culture give robust B cell but poor T cell reconstitution initially, the co-culture expanded cells gave significantly enhanced T cell reconstitution, reaching the normal T to B cell ratio as in humans and mice. The observed T cell-bias was opposite to the effect reported by Giassi *et al.* (Giassi *et al.*, 2008, Exp Biol Med (Maywood) *233*, 997-1012), further suggesting a critical role of growth factors in regulating differentiation in the co-culture. Given that no reduction in long-term repopulation capacity was observed using the culture methods described herein, the T cell-biased reconstitution suggests that MSCs also support progenitor cell expansion. HSCs cultured in the FCF culture in the presence of activin A gave a dramatically increased reconstitution of T cells in NSG mice, whereas adding the antagonist of activin A, follistatin, into the co-culture leads to reduction of T cell reconstitution in NSG mice. These results suggest that activin A secreted by MSCs stimulates lymphohematopoiesis in the co-culture by favoring T cell development.

The teachings of all patents, published applications and references cited herein are incorporated by reference in their entirety.

While this invention has been particularly shown and described with references to example embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. A method for expanding a population of hematopoietic stem cells (HSCs) comprising:

(a) co-culturing the HSCs with mesenchymal stem cells (MSCs) in the presence of angiopoietin-like 5 (Angplt5) growth factor, IGF-binding protein 2 (IGFBP2), stem cell factor (SCF), fibroblast growth factor (FGF), thrombopoietin (TPO), or a combination thereof, thereby producing a cell culture; and

(b) maintaining the cell culture under conditions in which an expanded population of HSCs is produced.

2. The method of Claim 1, wherein the method further comprises adding heparin to the cell culture.

3. The method of Claim 1, wherein the method further comprises adding an inhibitor of Activin A to the cell culture.

4. The method of Claim 1 wherein the MSCs express the Angptl5 growth factor.

5. The method of Claim 4, wherein the MSCs are genetically engineered to express the Angplt5 growth factor.

6. The method of Claim 5 wherein the Angplt5 growth factor is human Angplt5 growth factor.

7. The method of Claim 5, wherein the MSCs are genetically engineered with a viral vector or a non-viral vector to express the Angplt5 growth factor.

8. The method of Claim 7, wherein the viral vector is a lentiviral vector, an adenovirus vector, or a retrovirus vector.

9. The method of Claim 1, wherein the HSCs are freshly isolated HSCs, cryopreserved HSCs, or a combination thereof.

10. The method of Claim 1, wherein the HSCs are obtained from a single donor.

11. The method of Claim 1, wherein the HSCs are obtained from bone marrow, peripheral blood, umbilical cord blood, fetal liver, or a combination thereof.

12. The method of Claim 1, wherein the HSCs are human HSCs.

13. The method of Claim 1, wherein the MSCs are freshly isolated MSCs, cryopreserved MSCs, or a combination thereof.

14. The method of Claim 1, wherein the MSCs are obtained from bone marrow, umbilical cord blood, umbilical cord tissue, fetal tissue, embryonic stem cells, or a combination thereof.

15. The method of Claim 1, wherein the MSCs are human MSCs.

16. The method of Claim 1, wherein the HSCs and MSCs are obtained from the same donor.

17. The method of Claim 1, wherein the HSCs and the MSCs are added to the culture at an HSC to MSC ratio of 40:1, 20:1, 10:1. 1:1, 1:5, 1:10, 1:20, or 1:40.

18. The method of Claim 1, wherein one or more of the HSCs are in cell-to-cell contact with one or more of the MSCs.

19. The method of Claim 1, wherein the HSCs and MSCs are co-cultured for about 7 days, about 10 days, about 11 days, about 14 days, about 17 days, about 21 days, about 24 days, about 28 days, about 32 days, about 36 days, about 40 days, about 44 days, about 48 days, about 52 days, or more.

20. The method of Claim 1, wherein the HSCs and MSCs are co-cultured in serum-free medium.

21. The method of Claim 1, wherein the expanded population of HSCs are CD34+, CD133+, CD34+CD133+, or a combination thereof.

22. The method of Claim 1, further comprising monitoring the expansion of HSCs.

23. The method of Claim 22, wherein the expansion of HSCs is monitored by detecting the expression of CD34+, CD133+, CD34+CD133+, or a combination thereof, by the expanded population of HSCs.

24. The method of Claim 1, wherein the expanded population of HSCs is increased about 10 fold, about 20 fold, about 30 fold, about 40 fold, 50 fold, about 60 fold, about 70 fold, about 80 fold, about 90 fold, about 100 fold, about 110 fold, about 120 fold, about 130 fold, about 140 fold, about 150 fold, or more.

25. The method of Claim 24, wherein the expanded population of HSCs is increased about 60 fold in 11 days of co-culture.

26. The method of Claim 24, wherein the expanded population of HSCs is increased about 150 fold in 14 days of co-culture.

27. The method of Claim 24, wherein the expanded population of HSCs is increased about 10 fold in 10 days of co-culture, and wherein the HSCs are human HSCs.

28. The method of Claim 1, further comprising purifying the expanded population of HSCs.

29. The method of Claim 1, wherein the expanded population of HSCs are capable of stable, long term reconstitution of blood cells when transplanted into a mammal.

30. The method of Claim 1, wherein the expanded population of HSCs have severe combined immunodeficient (SCID) repopulating activity when transplanted into a mammal.

31. The method of Claim 1, wherein the expanded population of HSCs comprise severe combined immunodeficient (SCID) mouse repopulating cells (SRCs), and the SRCs are expanded about 80 fold, about 85 fold, about 90 fold, about 95 fold, about 100 fold, about 500 fold, about 1,000 fold, about 2,000 fold, about 4,000 fold, about 8,000 fold, about 12,000 fold, or more, within the expanded population of HSCs.

32. The method of Claim 31, wherein the SRCs are expanded about 80 fold in 11 days of co-culture.

33. The method of Claim 31, wherein the SRCs are expanded about 12,000 fold in 40 days of co-culture.

34. The method of Claim 12, further comprising transplanting the expanded population of human HSCs into a mammal.

35. The method of Claim 30, wherein the mammal is a mouse or a human.

36. The method of Claim 30, wherein the expanded population of human HSCs gives rise to reconstitution of multiple lineages of blood cells in the mammal.

37. The method of Claim 36, wherein the reconstitution of multiple lineages of blood cells in the mammal is a stable, long term reconstitution.

38. The method of Claim 36, wherein the mammal is an immunocompromised mammal.

39. The method of Claim 36, wherein the mammal that receives the expanded population of HSCs develop myeloid progenitor cells, mature hematopoietic cells, or a combination of cells thereof, wherein said cells are differentiated from the expanded population of HSCs.

40. The method of Claim 36, wherein the mammal that receives the expanded population of HSCs develop CD3+ cells, CD19+ cells, CD19+IgM+ cells, CD14+CD33+ cells, CD33+ cells, CD34+ cells, CD34+CD133+ cells, CD11c+ HLA-DR+ cells, CD56+ cells, CD235a+, CD235b+, CD235ab+,

CD41+, CD38+, CD45RA+, CD127+, CD90+, or a combination of cells thereof, which are cells differentiated from the expanded population of HSCs.

41. The method of Claim 33, wherein the HSCs transplanted into a mammal produce an enhanced CD3+ T cell reconstitution in the mammal which leads to an enhanced T cell response in the mammal as compared to a suitable control.

42. A method for expanding a population of hematopoietic stem cells (HSCs), wherein the method selectively inhibits the expansion HSCs that differentiate into CD3+ cells when transplanted into a mammal, the method comprising:

(a) co-culturing HSCs with mesenchymal stem cells (MSCs) in the presence of an inhibitor of Activin A, thereby producing a combination; and

(b) maintaining the combination under conditions in which an expanded population of HSCs are produced,

wherein the expanded population of HSCs when transplanted into a mammal differentiate into fewer CD3+ cells as compared to an expanded population of HSCs co-cultured with MSCs is the absence of an inhibitor of Activin A.

43. The method of Claim 42, wherein the HSCs and MSCs are cultured in the presence of angiopoietin-like 5 (Angptl5) growth factor, IGF-binding protein 2 (IGFBP2), stem cell factor (SCF), fibroblast growth factor (FGF), thrombopoietin (TPO), or a combination thereof.

44. The method of Claim 43, wherein the MSCs are genetically engineered to express Angptl5 growth factor.

45. The method of Claim 42, wherein the inhibitor of Activin A is follistatin.

46. The method of Claim 43, wherein the method further comprises adding heparin to the co-culture combination.

47. An expanded population of HSCs produced by the method of any one of Claims 1-32 and 42-46.

48. A mammal produced by the method of any one of Claims 33-41.

49. The method of Claim 1, wherein the culture of HSCs increases the population of HSCs at least about 40 fold, at least about 50 fold, at least about 60 fold, at least about 70 fold, at least about 80 fold, at least about 90 fold, at least about 100 fold, at least about 150 fold, at least about 250 fold, at least about 500 fold, at least about 1,000 fold, at least about 2,500 fold, at least about 5,000 fold, at least about 7,500 fold, at least about 10,000 fold, at least about 12,500 fold, at least about 15,000 fold, at least about 17,500 fold, at least about 20,000 fold, at least about 25,000 fold, at least about 30,000 fold, at least about 35,000 fold, or more, to produce an expanded population of HSCs.

50. The method of Claim 49, wherein the expanded population of HSCs are capable of self-renewal.

51. The method of Claim 1, further comprising removing at least about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of the expanded population of HSCs and further expanding the remaining HSCs in the co-culture.

52. A method for expanding a population of hematopoietic stem cells (HSCs) comprising:

(a) co-culturing a seed population of HSCs with mesenchymal stem cells (MSCs), wherein the MSCs are genetically engineered to express angiopoietin-like 5 (Angplt5) growth factor, thereby producing a cell culture; and

(b) maintaining the cell culture under conditions in which the population of HSCs is expanded 50 fold or more, as compared to the seed population of HSCs.

53. The method of Claim 52, wherein the method further comprises adding IGF-binding protein 2 (IGFBP2), stem cell factor (SCF), fibroblast growth factor (FGF), thrombopoietin (TPO), heparin, or a combination thereof, to the cell culture.

54. The method of Claim 53, wherein the method further comprises adding an inhibitor of Activin A to the cell culture.

55. A method for expanding a population of hematopoietic stem cells (HSCs) comprising:

(a) culturing a seed population of HSCs in the presence of three or more growth factors selected from the group consisting of angiopoietin-like 5 (Angplt5) growth factor, IGF-binding protein 2 (IGFBP2), stem cell factor (SCF), fibroblast growth factor (FGF), and thrombopoietin (TPO), thereby producing a cell culture; and

(b) maintaining the cell culture under conditions in which the number of HSCs is expanded at least 50 fold as compared to the number of HSCs present in the seed population of HSCs.

56. The method of Claim 55, wherein the method further comprises adding Activin A, heparin, or a combination thereof, to the cell culture.

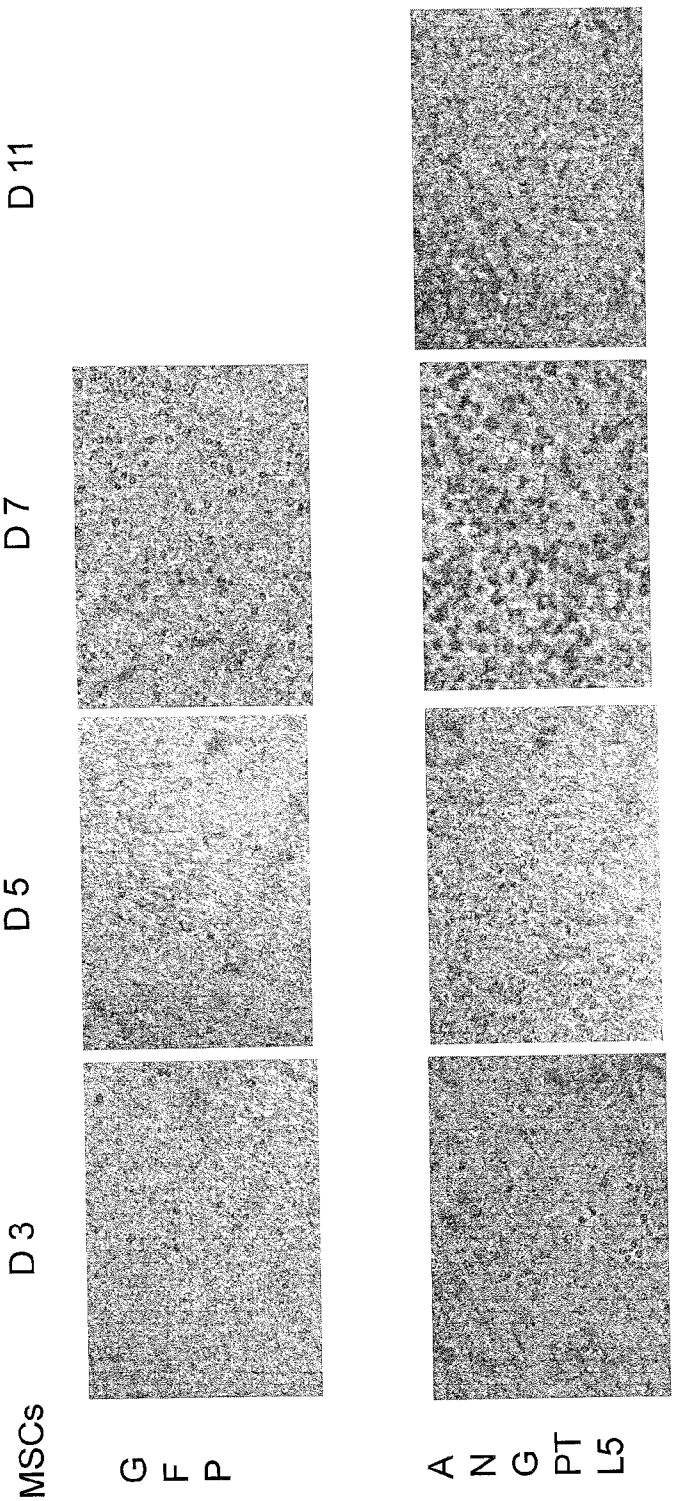


FIG. 1

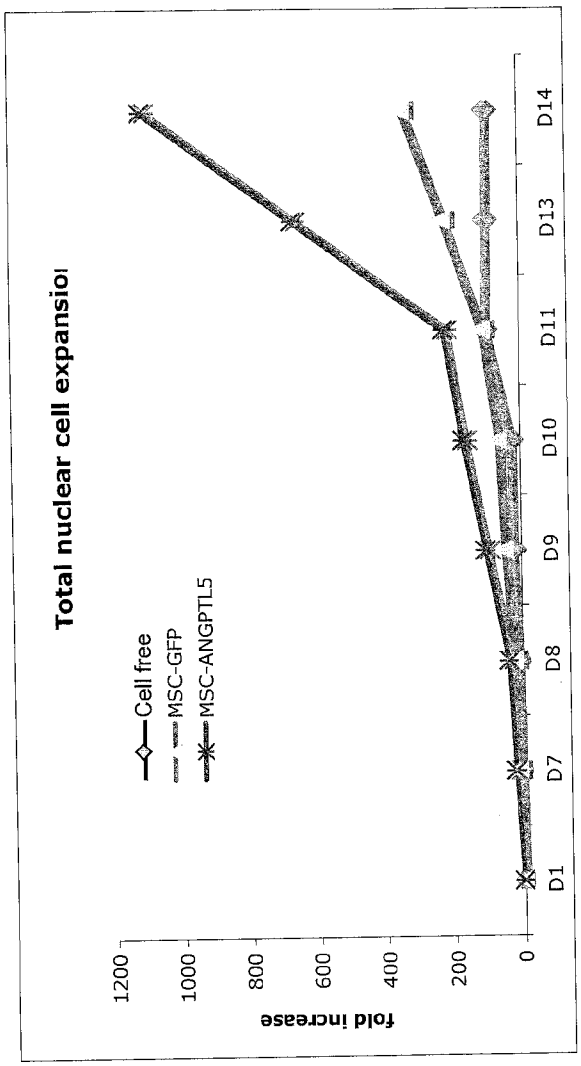

Total nuclear cell expansio
Cell free
MSC-GFP
MSC-ANGPTL5
fold increase
0
200
400
600
800
1000
1200
D1
D7
D8
D9
D10
D11
D13
D14

FIG. 2A

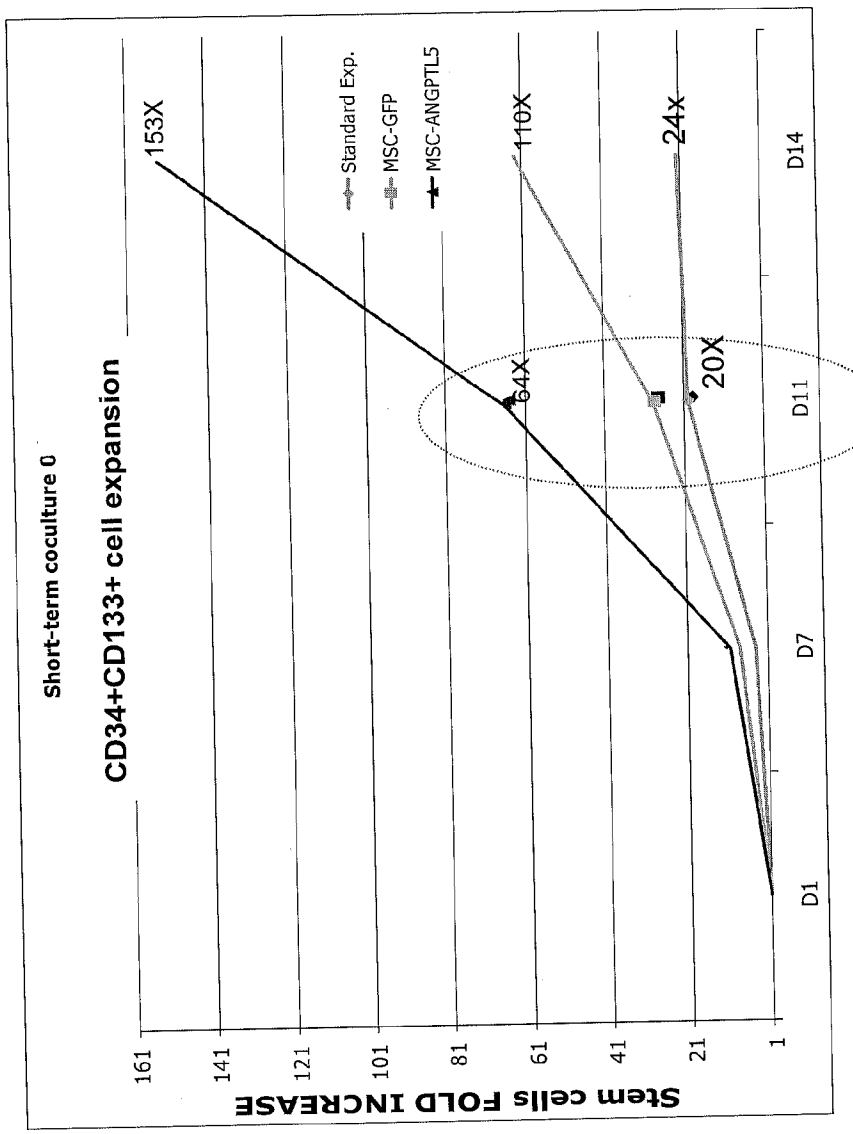

Short-term coculture 0
CD34+CD133+ cell expansion
153X
110X
64X
24x
20X
Standard Exp.
MSC-GFP
MSC-ANGPTL5
Stem cells FOLD INCREASE
161
141
121
101
81
61
41
21
1
D1
D7
D11
D14

FIG. 2B

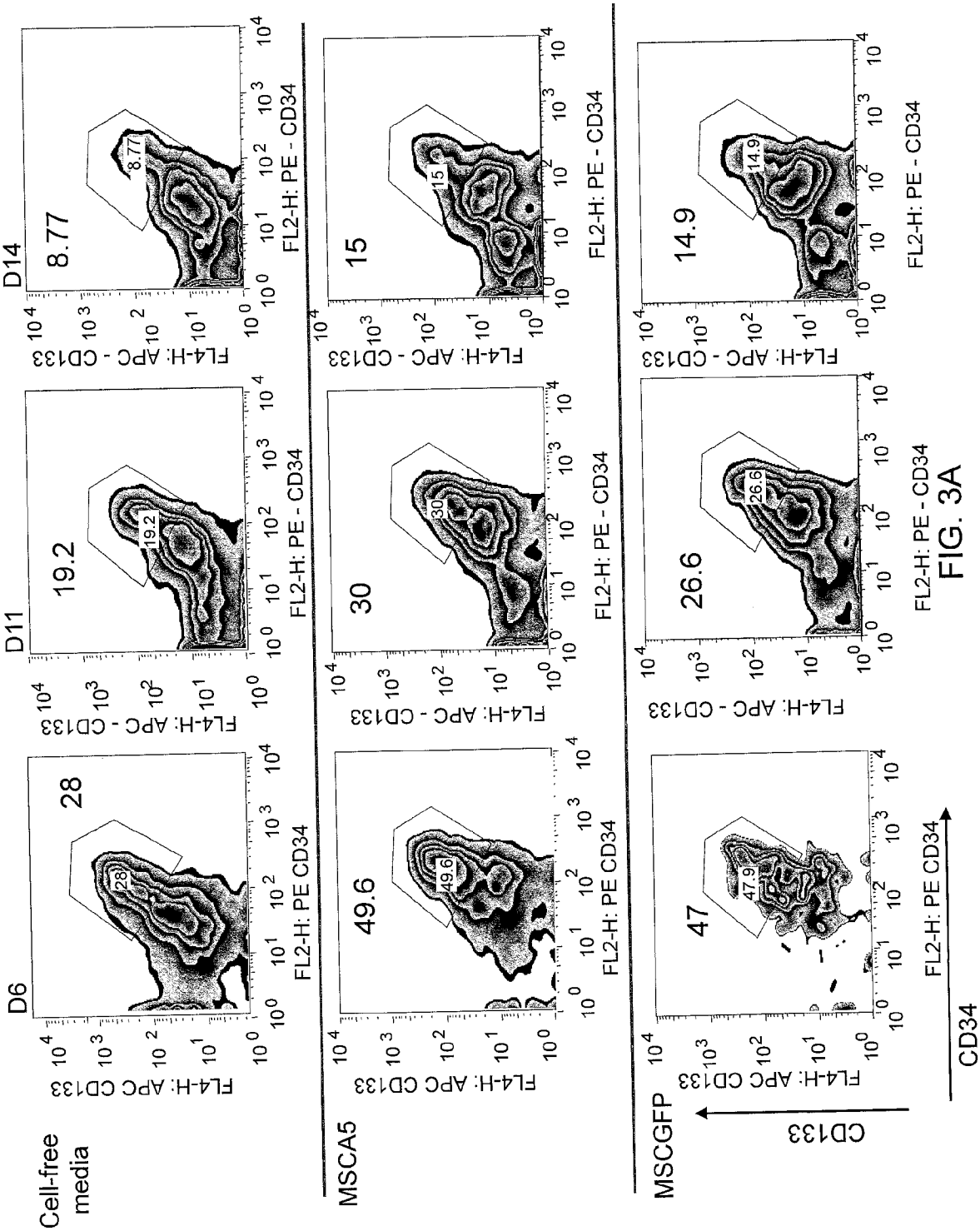

Cell-free media
MSCA5
MSCGFP
D6
D11
D14
28
19.2
8.77
49.6
30
15
47
26.6
14.9
FL4-H: APC CD133
FL2-H: PE CD34
CD133
CD34

FIG. 3A

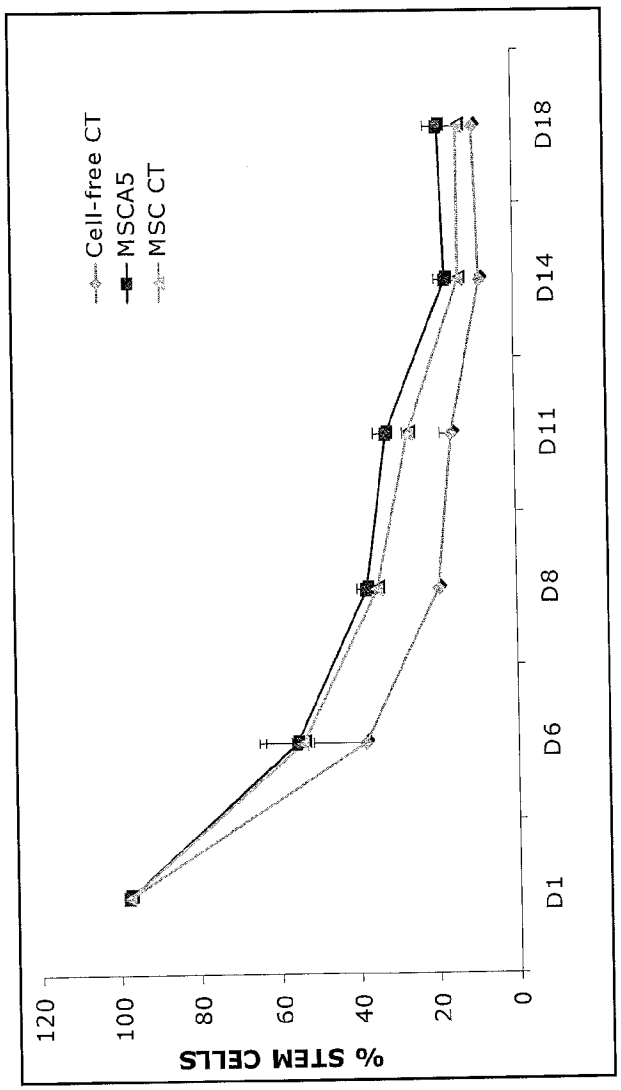

Cell-free CT
MSCA5
MSC CT
% STEM CELLS
120
100
80
60
40
20
0
D1
D6
D8
D11
D14
D18

FIG. 3B

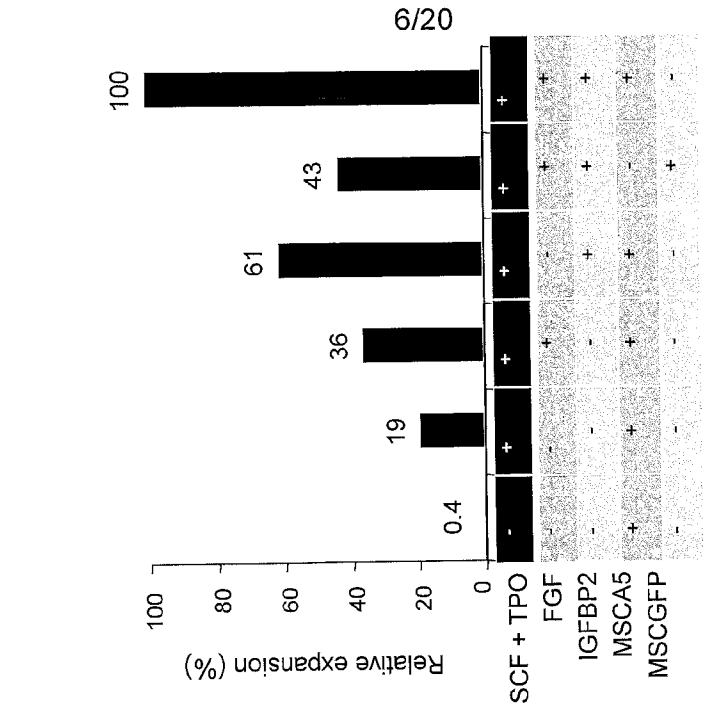


FIG. 5

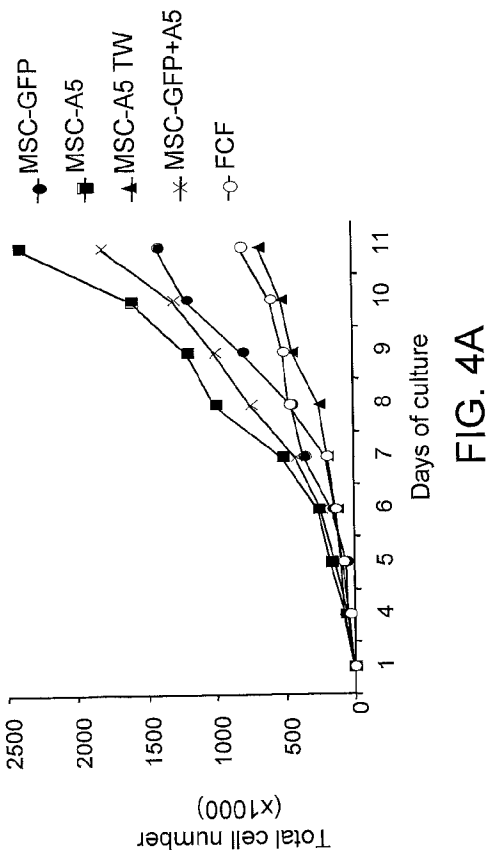


FIG. 4A

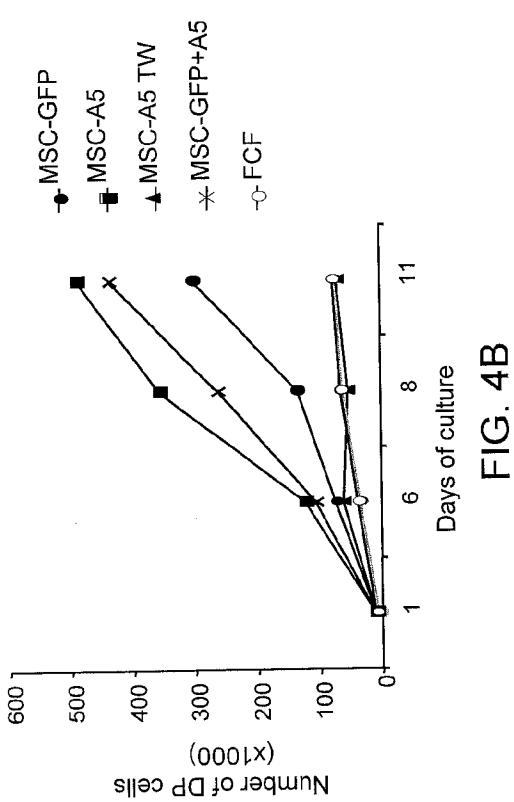


FIG. 4B

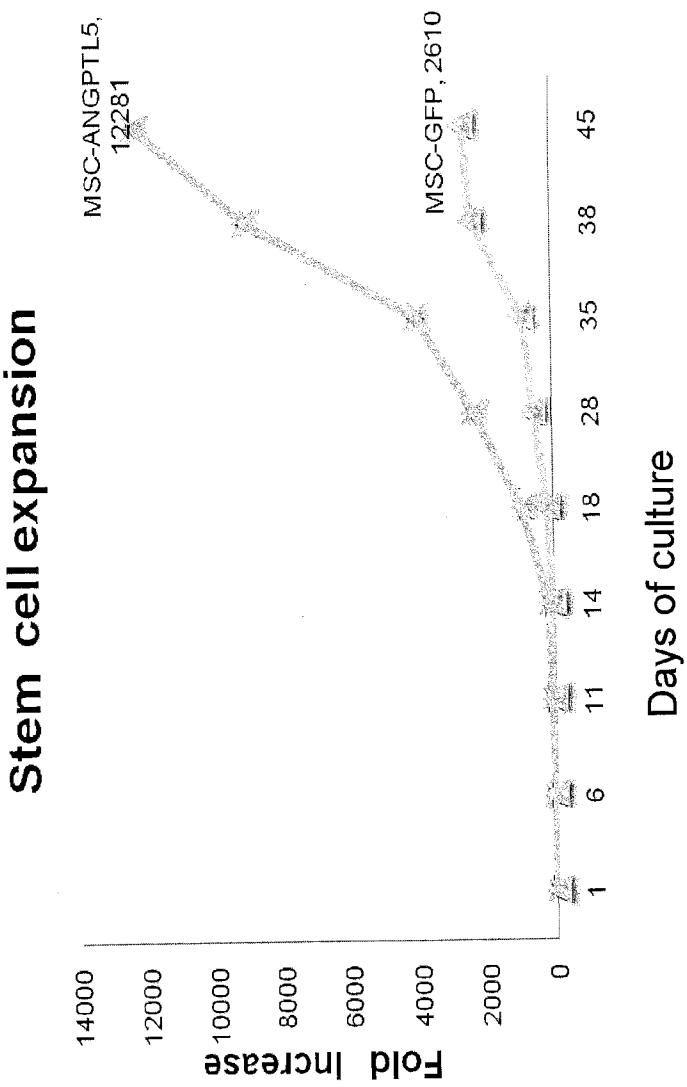

Stem cell expansion
Fold Increase
14000
12000
10000
8000
6000
4000
2000
0
1
6
11
14
18
28
35
38
45
Days of culture
MSC-ANGPTL5, 12281
MSC-GFP, 2610

FIG. 6

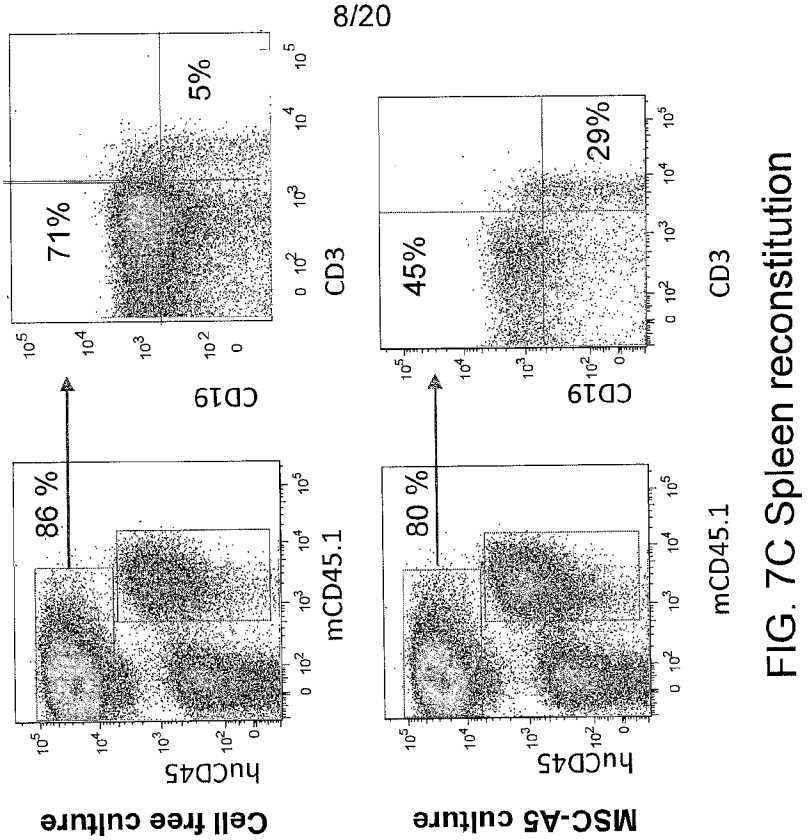


FIG. 7B Blood reconstitution

FIG. 7C Spleen reconstitution

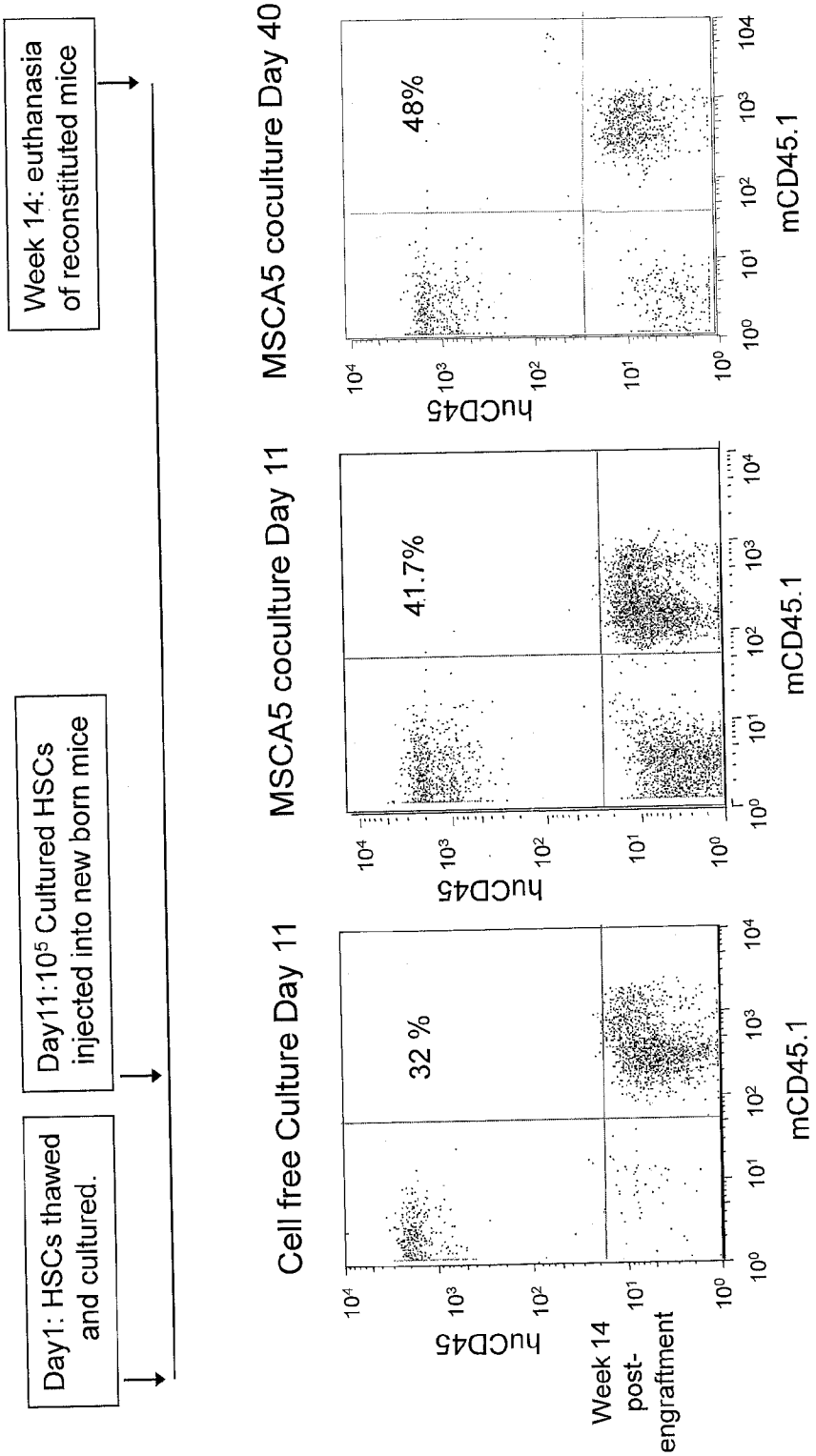


FIG. 7 A Experiment timeline

FIG. 8A

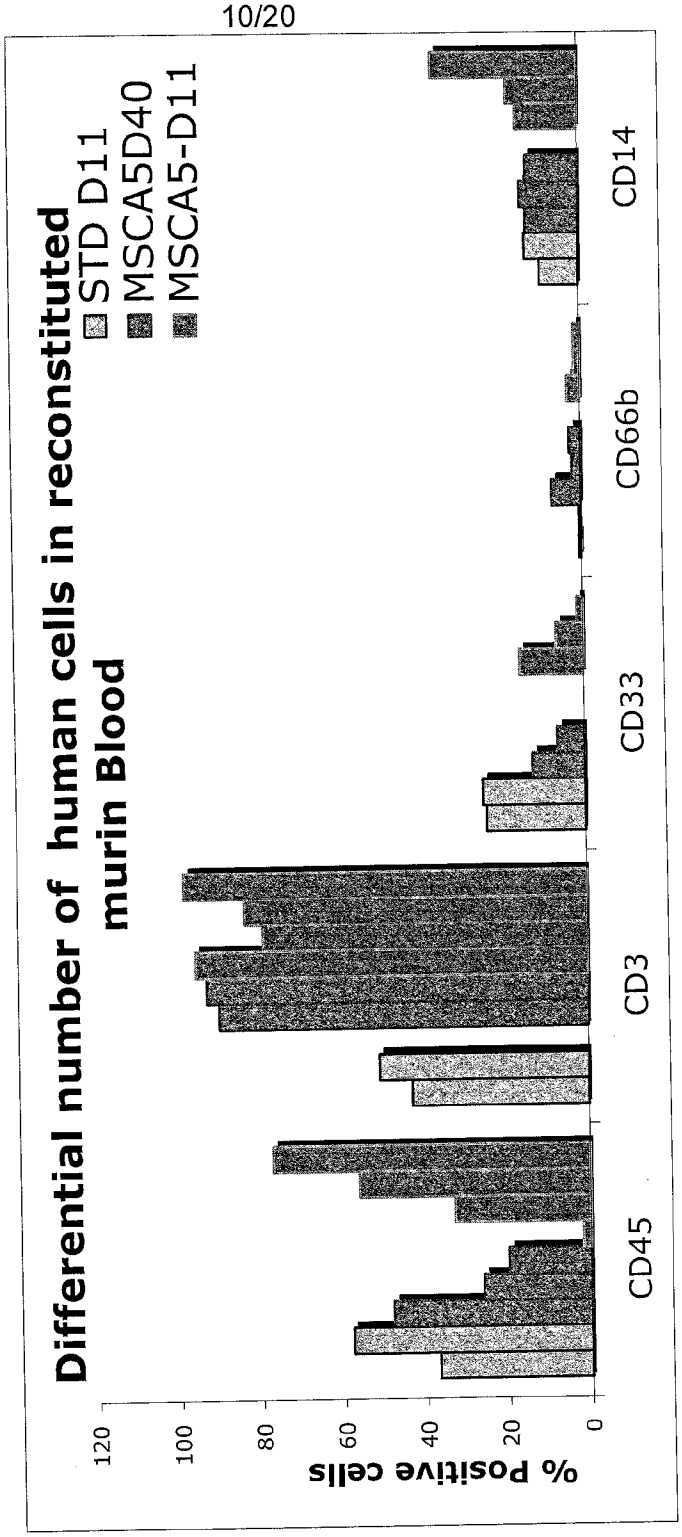

Differential number of human cells in reconstituted murin Blood
STD D11
MSCA5D40
MSCA5-D11
% Positive cells
120
100
80
60
40
20
0
CD45
CD3
CD33
CD66b
CD14

FIG. 8B

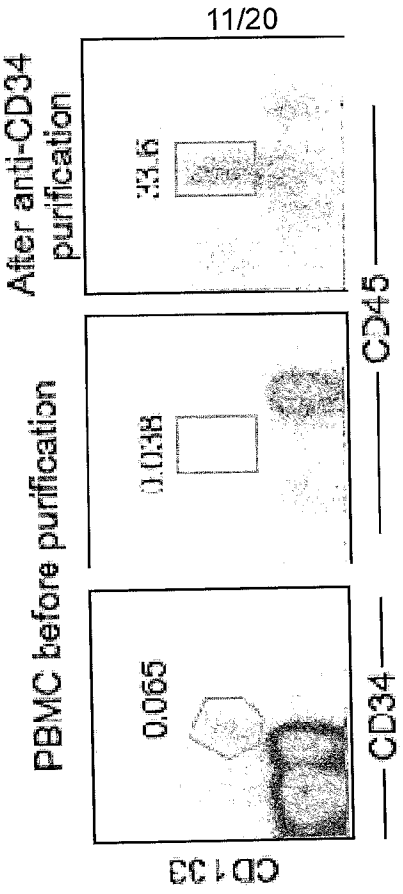

11/20
After anti-CD34 purification
PBMC before purification
0.065
CD133
CD34
CD45

FIG. 9B

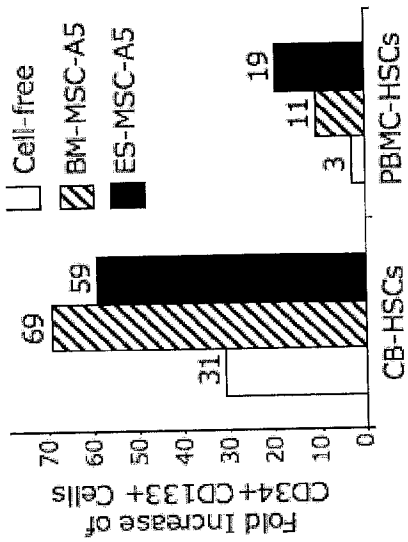

Cell-free
BM-MSC-A5
ES-MSC-A5
Fold Increase of CD34+CD133+ Cells
0
10
20
30
40
50
60
70
69
59
31
19
11
3
CB-HSCs
PBMC-HSCs

FIG. 9A

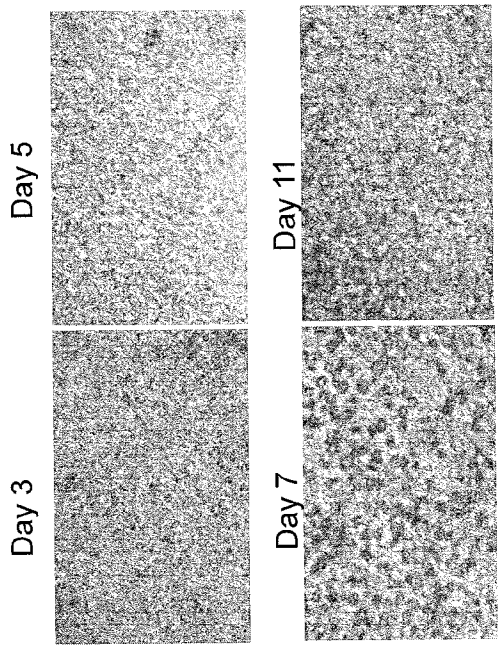


FIG. 10 A

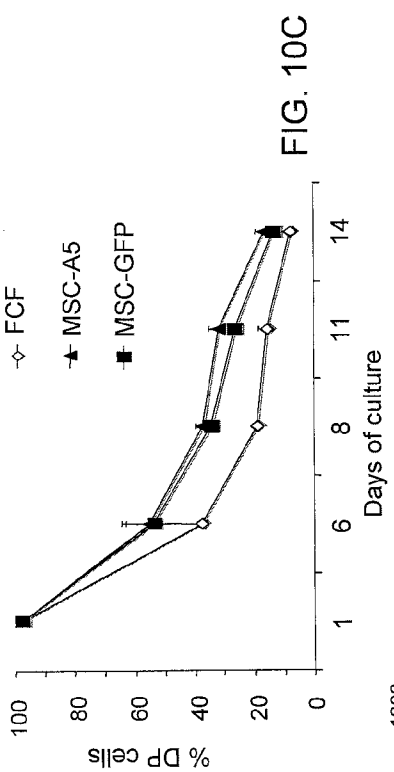


FIG. 10C

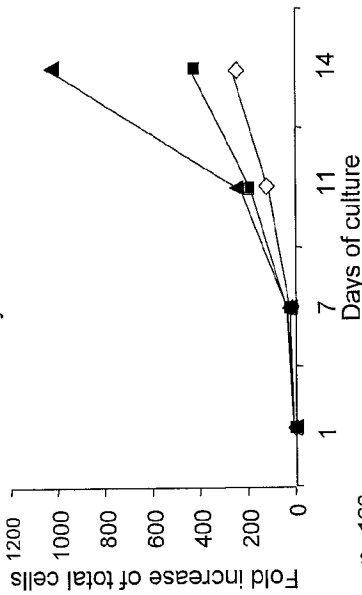


FIG. 10D

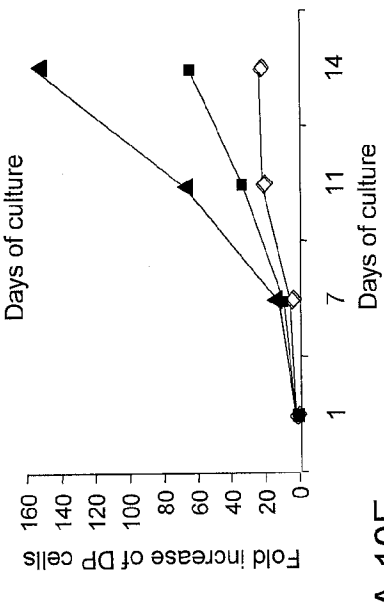


FIG. 10E

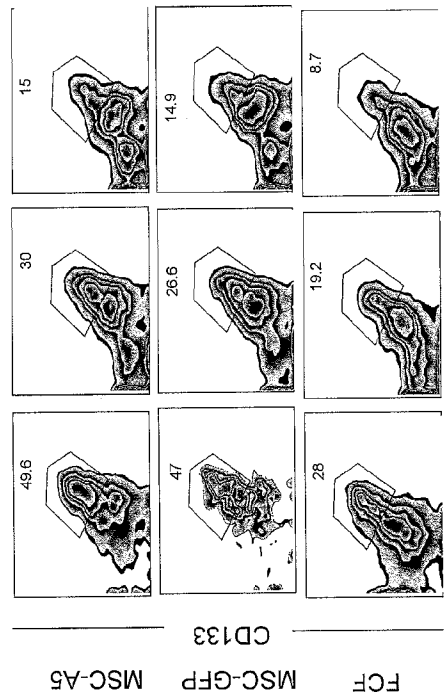


FIG. 10B

FIGS. 10A-10E

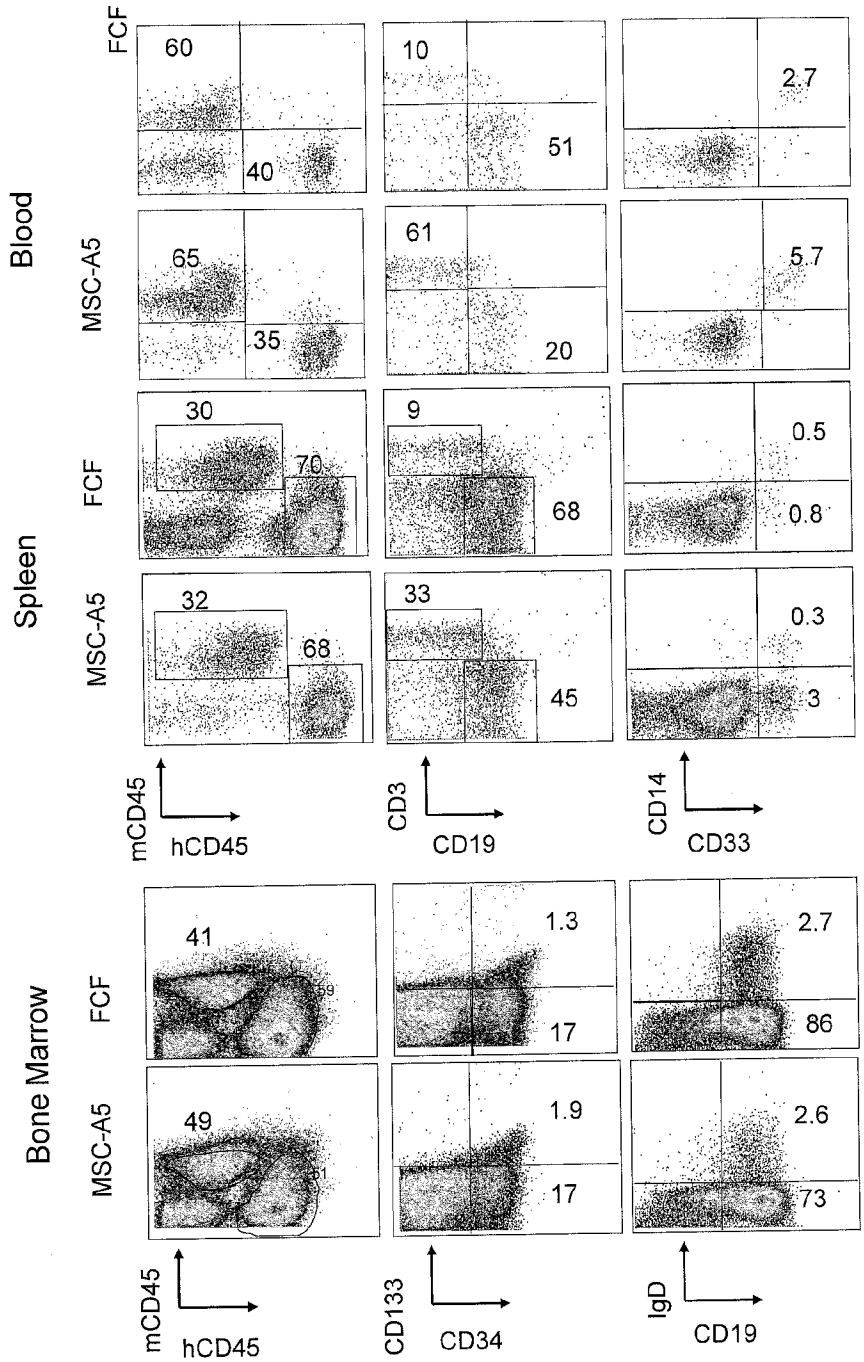

Blood
Spleen
Bone Marrow
FCF
MSC-A5
60
40
10
51
2.7
65
35
61
20
5.7
30
70
9
68
0.5
0.8
32
68
33
45
0.3
3
41
49
1.3
17
1.9
17
2.7
86
2.6
73
mCD45
hCD45
CD3
CD19
CD14
CD33
CD133
CD34
IgD
CD19

FIG. 11A

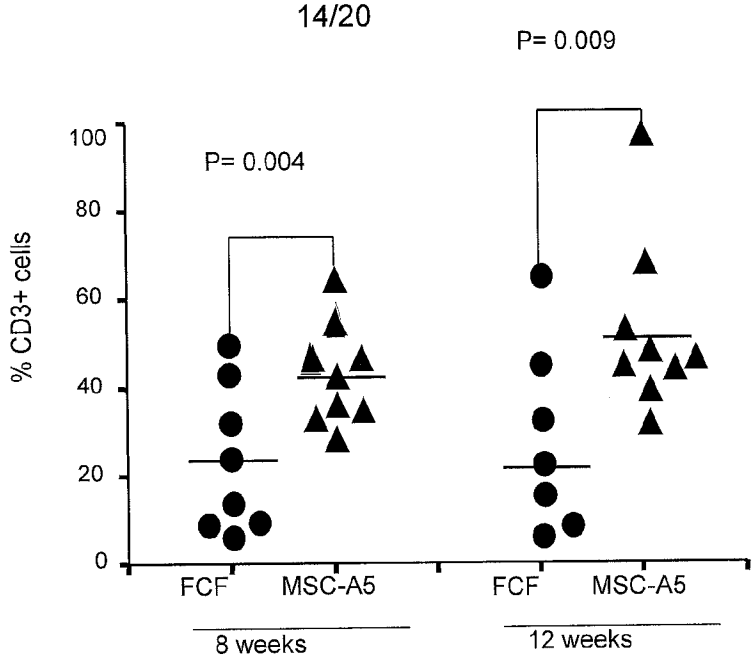


FIG. 11B

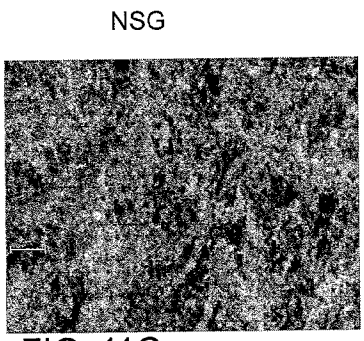


FIG. 11C

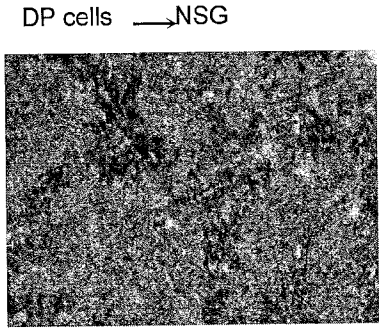


FIG. 11D

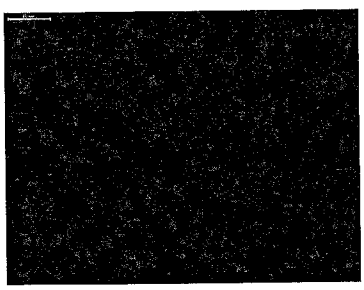


FIG. 11E

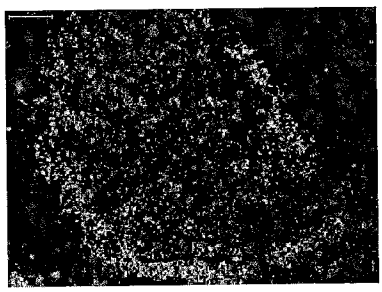


FIG. 11F

DAPI CD20 CD3

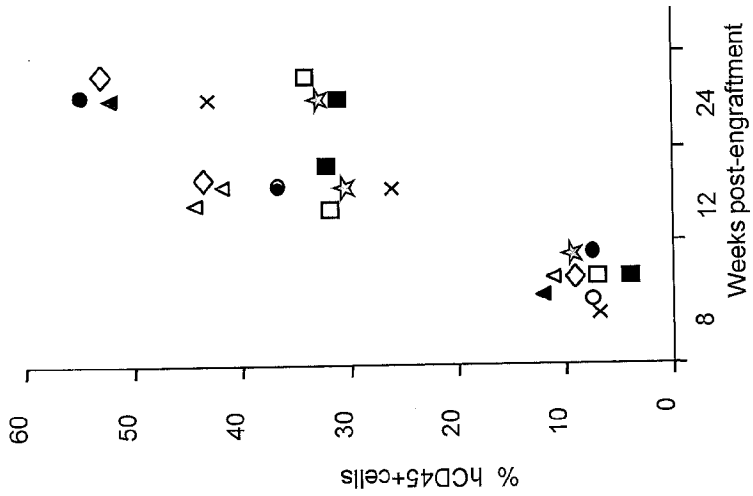


FIG. 12B

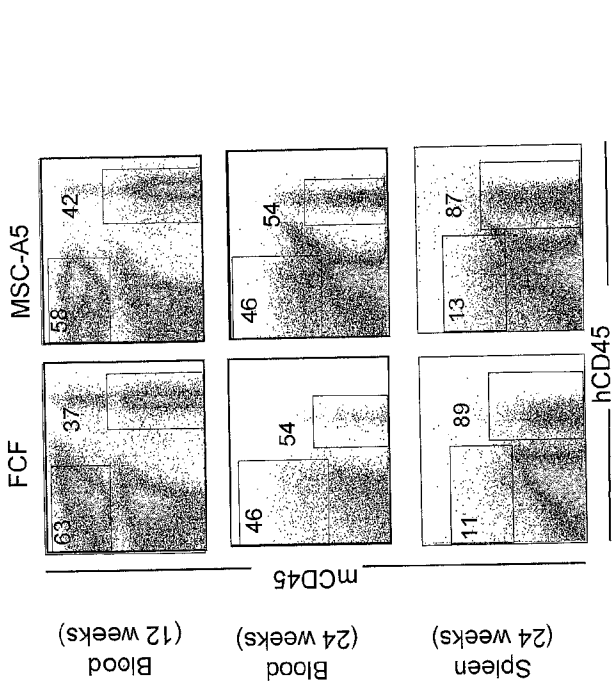


FIG. 12A

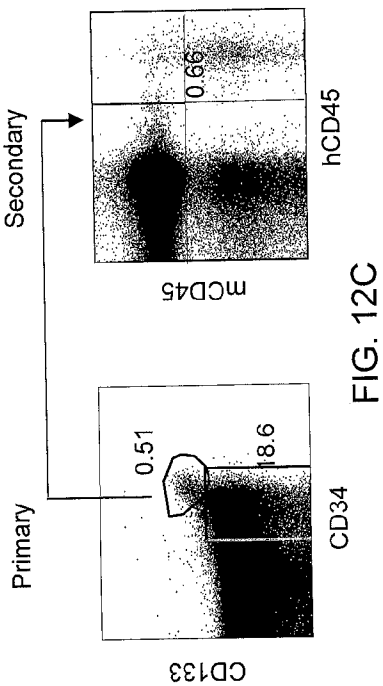


FIG. 12C

FIGS. 12A-12C

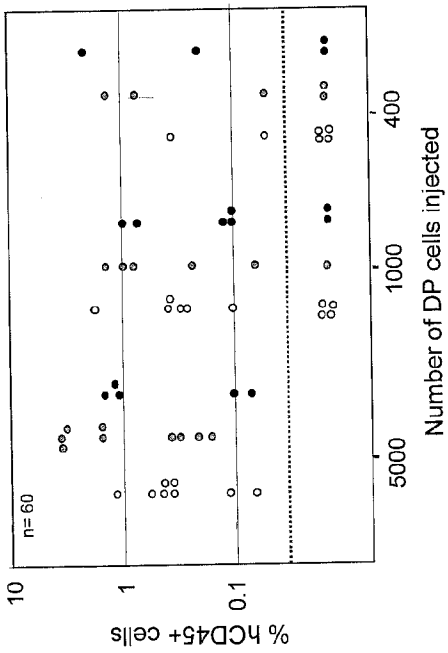


FIG. 13A

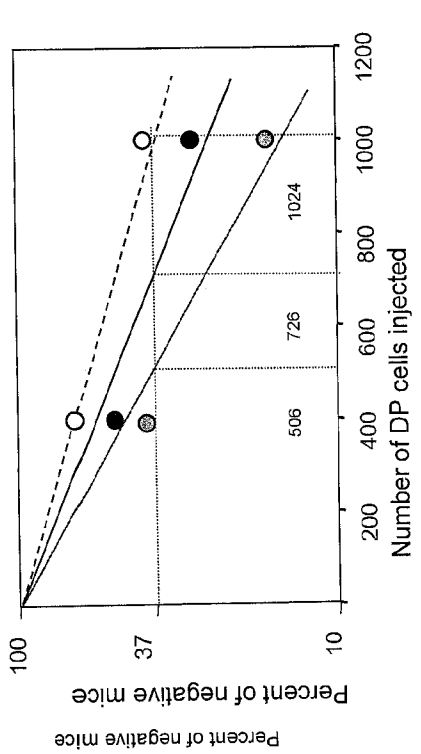


FIG. 13B

FIGS. 13A-13C

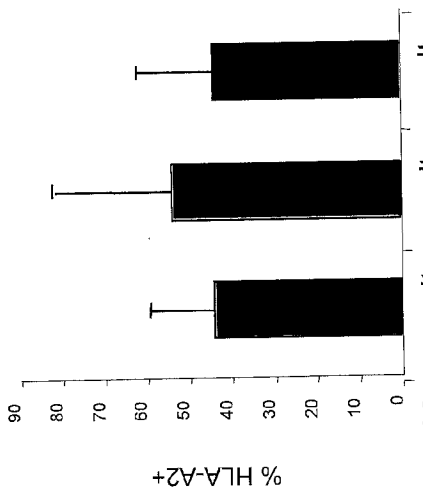


FIG. 13C

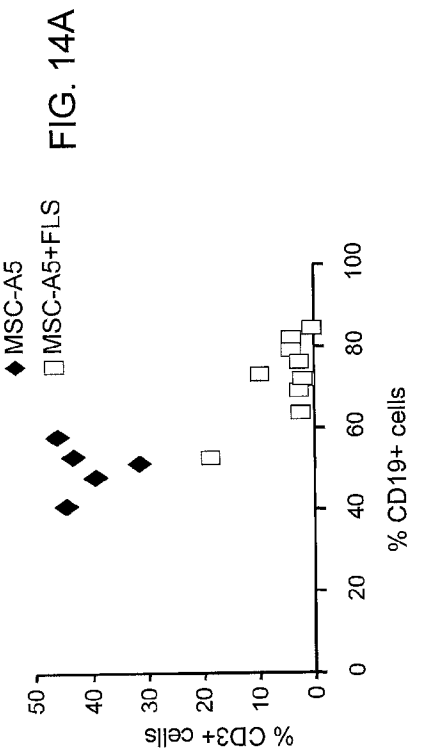


FIG. 14A

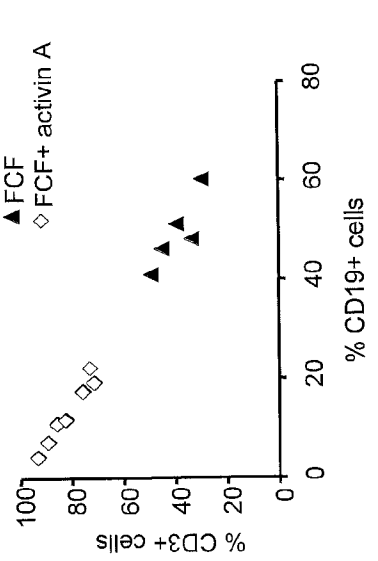


FIG. 14B

FIGS. 14A-14B

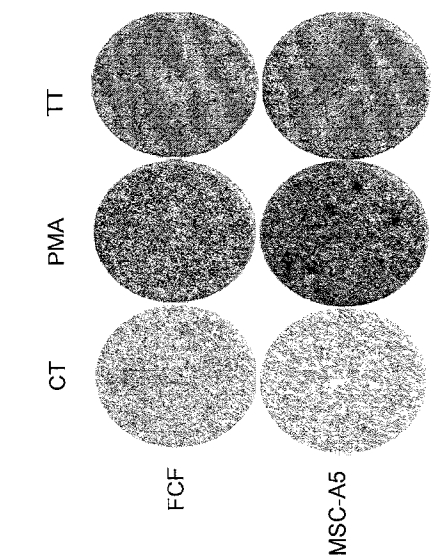


FIG. 15A

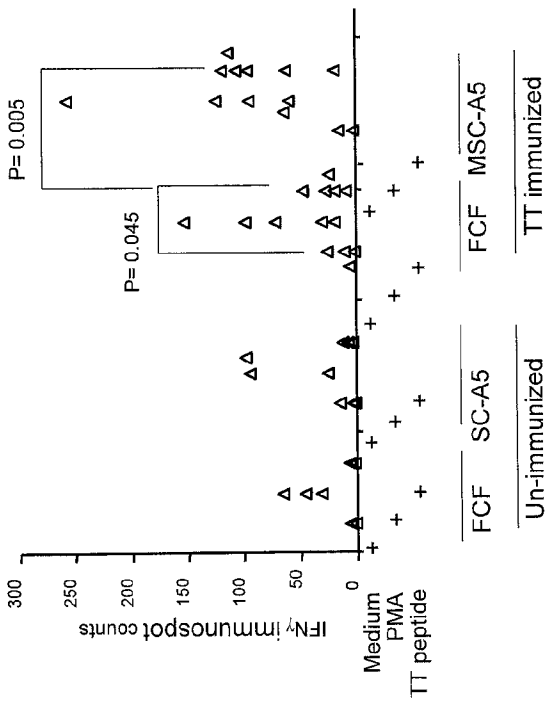


FIG. 15B

FIGS. 15A-15B

	hCD45+		CD33+		CD3+		CD19+		CD33+CD14+		CD11c+HLA-DR+		CD15+	
	Median	(Range)	Median	(Range)	Median	(Range)	Median	(Range)	Median	(Range)	Median	(Range)	Median	(Range)
Blood														
FCF	40	(33-50)	5.6	(3-8)	11.5	(10-44)	36	(21-51)	4.6	(1.5-5.2)	5	(4.7-5.9)	5.67	(3.9-6.4)
MSC.A5	34	(26-46)	5.7	(3-9.2)	44	(21-68)	21	(7-54)	5.63	(3.8-9.7)	8.9	(3.2-16.5)	9.69	(3.9-12.7)
Spleen														
FCF	80	(76-87)	1.1	(0.4-1.1)	17.18	(5.3-29)	54	(20-87)	0.13	(0.07-0.19)	1	(1-2.9)	1	(0.7-1)
MSC.A5	75	(63-87)	1	(0.8-3.2)	30	(8.3-54)	55	(25.8-85)	0.35	(0.2-1.1)	1.44	(0.7-2.3)	0.85	(0.5-1.5)
BM														
FCF	44	(41-48)	3.35	(3-5.2)	2.68	(0.4-4.9)	69.5	(60-79)	1.9	(1-2.1)	1.3	(1.2-2)	3.7	(1.5-6)
MSC.A5	45	(19-74)	4.7	(2.1-9.8)	2.57	(0.8-7.8)	72.3	(67-76)	1.8	(1.3-4.6)	1.5	(1.2-4.2)	2.33	(0.4-4.6)

FIG. 16

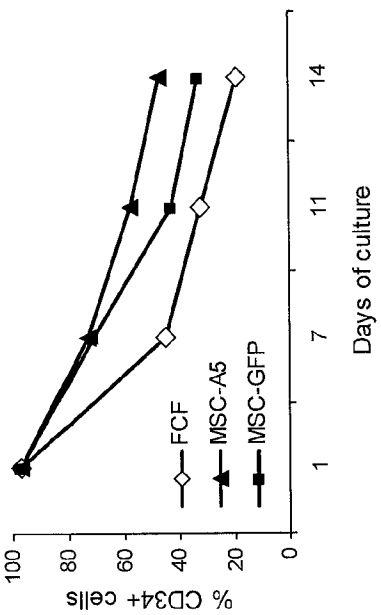


FIG. 17A

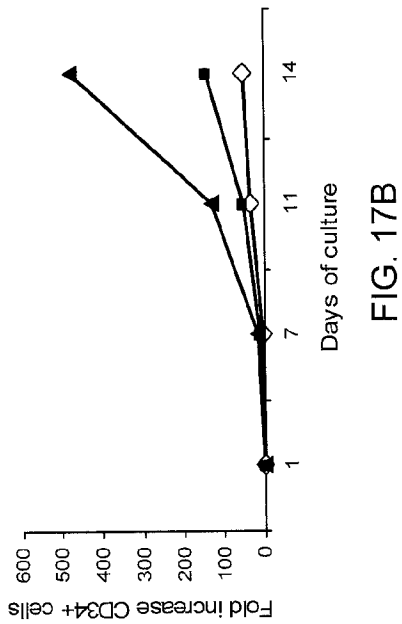


FIG. 17B

FIGS. 17A-17B

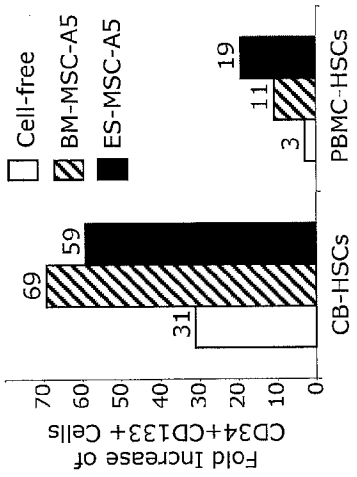


FIG. 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/36664

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 5/0789
USPC - 435/372, 435/404
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)
USPC - 435/372, 404

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/366,363,325

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWest (PGPB, USPT, EPAB, JPAB)
Search Terms Used: (HEMATOPOIETIC OR MULTIPOTENT OR HSC) AND (STROMA OR MESENCHYMAL OR MSC) AND ("ANGIOPOIETIN-LIKE 5" OR ANGPTL5) AND (IGFBP2 OR SCF OR FGF OR TPO) AND (FOLLISTATIN OR FSTL-3); ((PROPAGAT$3

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2007/0020757 A1 (Zhang et al.) 25 January 2007 (25.01.2010), especially: abstract, para[0006], para[0007], para[0009], para[0010], para[0011], para[0018], para[0019], para[0029], para[0041], para[0048], para[0051], para[0053], para[0055], para[0057], para[0058], para[0067], para[0063], para[0064], para[0071], para[0072], para[0072], para[0082], para[0091], para[0093], para[0111], para[0112]	1, 2, 9-16, 19-23, 28-29, 34, 48, 51 --- 3-8, 17-18, 24-27, 30-33, 35-47, 49-50, 52-56
Y	US 2007/0275895 A1 (Duan et al.) 29 November 2007 (29.11.2007), the entire document, especially para[0035], para[0003], para[0006], para[0228], para[0273], para[0372]	3, 42-46, 54
Y	US 2002/0164699 A1 (Shimkets et al.) 7 November 2002 (07.11.2002), the entire document, especially abstract, para[0007], para[0008] para[0187], para[0198], para[0200], para[0201]	4-8, 44
Y	ZHANG et al., Co-culture of Umbilical Cord Blood CD34+ Cells with Human Mesenchymal Stem Cells, Tissue Eng. Vol. 12, No. 8 p.2161-2170 (2006), the whole document, especially p.2162, col. 2	17-18, 30, 35
Y	WO 2006/113881 A2 (McNiece et al.) 26 October 2006 (26.12.2006), the entire document, especially p.3, ln.5-12, p.12, ln.30 to p.13, ln.8-15; p.41, ln 7-10, p.42, ln.34 to p.43, ln.32; p.43, ln.13-33; p.44, ln.14-26;	24-27, 31-33, 36-41, 47, 49-50, 52- 56

☐ 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	Date of mailing of the international search report
8 July 2010 (08.07.2010)	30 JUL 2010
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: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)