IN VITRO TECHNIQUES FOR USE WITH STEM CELLS

CROSS-REFERENCES TO RELATED APPLICATIONS

The present application claims priority from:

(a) US Provisional Patent Application 60/576,266, filed June 1, 2004, entitled, "In vitro techniques for use with stem cells," and

(b) US Provisional Patent Application 60/588,520, filed July 15, 2004, entitled, "Indications for stem cell use."

Both of these applications are assigned to the assignee of the present patent application, and are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates generally to methods and apparatus for treatment of human patients suffering from vascular disorders, and specifically to methods and apparatus for facilitating angiogenesis and/or neovascularization and/or vasculogenesis.

BACKGROUND OF THE INVENTION

Some arterial dysfunction occurs due to narrowing of the arteries by fatty deposits or other vascular abnormalities. This may interfere with blood flow and/or prevent tissues and organs from being supplied with sufficient nutrients and oxygen.

Vascular disorders are common conditions and can severely compromise a patient's quality of life. Despite considerable advances in medical therapy and improvements in revascularization procedures for artery dysfunction, such as coronary artery graft, balloon angioplasty and stenting of the coronary vessels, a substantial proportion of patients suffer from artery dysfunction-derived disease.

Endothelial progenitor cells (EPCs) have been used to treat patients suffering from vascular diseases. In such severe cases, when drugs or direct revascularization procedures are not effective anymore, or cannot be used, alternative therapies are required. EPCs have been applied to ischemic tissue. EPCs have the ability to

differentiate in order to form endothelium, the layer of cells forming blood vessels. These cells are involved in re-endothelialization, neovascularization, vasculogenesis and angiogenesis processes. The mechanisms by which implanted EPCs can become part of the healing process include self-repopulation, fusion with cells of the injured tissue and secretion of cytokines and growth factors. Repopulation of EPCs and their differentiation into mature endothelial cells enables their functions in re-endothelialization, neovascularization, vasculogenesis and angiogenesis processes. Recent evidence suggests that fusion of EPCs with cells of injured tissue enhances tissue function regeneration. Moreover, following secretion of cytokines and growth factors, EPCs can influence cellular survival of cells inherent to the tissue, and may help the mobilization of stem cells to the injured tissue.

A common ancestor cell, the hemangioblast, gives rise to both endothelial and hematopoietic (blood cell) precursors. This ancestor cell differentiates into hematopoietic stem cells and angioblasts, which are mesodermal precursor cells, differentiating into endothelial precursors. These cells have the capacity to proliferate, migrate, and differentiate into endothelial cells, but have not yet acquired specific mature endothelial markers. Following commitment to the endothelial lineage, angioblasts assemble into a primitive vascular plexus of veins and arteries, in a process called vasculogenesis. This primitive vasculature is subsequently refined into a functional network by angiogenesis and by remodeling and arteriogenesis of newly formed vessels. EPCs have been shown to mobilize (i.e., migrate in increased numbers from the bone marrow (BM) into the circulation) in patients with vascular trauma or Acute Myocardial Infarction (AMI) (See, for example, the following two articles which are incorporated herein by reference: (a) Gill, M., S. Dias, et al. (2001), "Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells," Circ Res 88(2): 167-74; and (b) Shintani, S., T. Murohara, et al. (2001), "Mobilization of endothelial progenitor cells in patients with acute myocardial infarction," Circulation 103(23): 2776-9.) In general, the use of EPCs aims to promote the formation of natural bypasses within the ischemic or scarred tissue and thus alleviate the clinical condition of these patients.

Numerous animal experiments and clinical trials have investigated the potential of this therapy to augment blood flow and yield an associated alleviation of ischemic symptoms, as manifested by a patient's improvement in physical functioning.

Various sources for autologous EPCs for transplantation have been described, including stem cells aspirated directly from the bone marrow (BM), and BM-derived peripheral blood stem cells.

Progenitor cells, or stem cells, include bone marrow cells that can multiply, migrate and differentiate into a wide variety of cell types. Bone marrow hematopoietic stem cells are characterized as being "CD34 positive" (CD34+), i.e., expressing the CD34 marker.

It is assumed that the plasticity of well-defined populations of hematopoietic progenitors allows them to trans-differentiate in response to the environmental cues present in the target organ, and, more specifically, to convert into endothelial cells.

Transplantation of bone marrow is clinically appealing because of the relative simplicity of the medical procedure. It entails aspiration of bone marrow from the iliac crest and immediate re-injection of the aspirate or selected cells into the post-infarction scar. Nevertheless, the procedure is invasive and must be done under anesthesia.

The first evidence indicating the presence of EPCs in the adult circulation was obtained when mononuclear blood cells from healthy human volunteers were shown to acquire an endothelial cell-like phenotype in vitro and to incorporate into capillaries in vivo. (See Asahara, T., T. Murohara, et al. (1997), "Isolation of putative progenitor endothelial cells for angiogenesis," Science 275(5302): 964-7, which is incorporated herein by reference.) These putative EPCs were characterized via expression of CD34 and vascular endothelial growth factor receptor-2 (VEGFR-2/KDR), two antigens shared by embryonic endothelial progenitors, and hematopoietic stem cells (HSCs). In addition to CD34, early hematopoietic progenitor cells express CD133 (AC133), which is not expressed after differentiation. Currently, the widely accepted definition of EPCs in circulation is, for practical purposes, CD34+/VEGFR-2+ or CD133+/VEGFR-2+ cells.

Peripheral blood EPCs can be obtained from blood of untreated patients or from patients treated in order to augment EPC mobilization using cytokines such as granulocyte-colony stimulating factor (G-CSF), granulocyte monocyte colony-

stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF). The mobilization treatments are typically avoided in patients who suffer from hematological and arterial derived disorders. Treatments such as HMG CoA reductase inhibitors (statins) have also been reported to elevate numbers of EPCs in circulation. See, for example:

1. Dimmeler S., et al. (2001), "HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway," J. Clin. Invest. 108: 391-397.

2. Hyun-Jae, Hyo-Soo Kim, et al. (2003), "Effects of intracoronary infusion of peripheral blood stem-cells mobilized with granulocyte-colony stimulating factor on left ventricular systolic function and restenosis after coronary stenting in myocardial infraction: the MAGIC cell randomized clinical trial," The Lancet 363: 751-756.

3. Brigit Assmus, Volker Schachinger et al., (2002), "Transplantation of progenitor cells and regeneration enhancement in acute myocardial infraction (TOPCARE-AMI)," Circulation 106: 3009-3017.

4. Alexandra Aicher, Winfreid Brenner, et al., (2003), "Assessment of the tissue distribution of transplanted human endothelial progenitor cells by radioactive labeling," Circulation 107: 2134-2139.

Each of these articles is incorporated herein by reference.

The procedure for removing peripheral blood is simpler and more convenient for the patient than BM removal. The fact that EPCs can be isolated from peripheral blood is an additional important factor in the choice of using these cells for therapy. The isolation of progenitor cells from BM, which contains many more cell types, is technically more challenging, as well.

Results from EPC and BMC treatments show improved cardiac function, greater capillary density, marked increase in number of collateral vessels, improvement of echocardiographic left ventricular ejection fraction, decrease in ischemic area scarring and prevention of cardiomyocyte apoptosis in rat models of myocardial infarction. Furthermore, improved blood flow and capillary density and reduced rate of limb loss in hindlimb was shown in an ischemia model in nude mice.

The following articles, which are incorporated herein by reference, describe techniques which may be used in combination with techniques described herein:

(1) Kalka, C., H. Masuda, et al. (2000). "Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects." Circ Res 86(12): 1198-202.

(2) Kawamoto, A., H. C. Gwon, et al. (2001). "Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia." Circulation 103(5): 634-7.

(3) Kawamoto, A., T. Tkebuchava, et al. (2003). "Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia." Circulation 107(3): 461-8.

(4) Kamihata, H., H. Matsubara, et al. (2001). "Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines." Circulation 104(9): 1046-52.

(5) Kocher, A. A., M. D. Schuster, et al. (2001). "Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function." Nat Med 7(4): 430-6.

The following articles and book chapter, which are also incorporated herein by reference, describe techniques which may be used in combination with techniques described herein:

Flammera J et al. (2002). "The impact of ocular blood flow in glaucoma." Progress in Retinal and Eye Research 21:359-393.

Zarbin MA (2004). "Current concepts in the pathogenesis of age-related macular degeneration." Arch Ophthalmol. 122(4):598-614.

Frank RN (2004). "Diabetic retinopathy." N Engl J Med 350:48-58.

Singleton JR (2003). "Microvascular complications of impaired glucose tolerance." Diabetes 52:2867-2873.

Bahlmann FH et. (2004). "Erythropoietin regulates endothelial progenitor cells." Blood 103(3):921-6.

Greenfield, Ed. (2001). "Surgery: scientific principles and practice." Lippincot: Philadelphia, chapter 107.

Kouwenhoven EA et al. (2000). "Etiology and pathophysiology of chronic transplant dysfunction." Transplant Internat. 13(6):385-401.

Browne EZ et al. (1986). "Complications of skin grafts and pedicle flaps." Hand Clin. 2:353-9.

Chen et al. (1991). "Four types of venous flaps for wound coverage: a clinical appraisal." J. Trauma 31(9):1286-93.

Beatrice et al. (2004) Dermatol. Surg. 30(3):399.

Ferretti et al. (2003). "Angiogenesis and nerve regeneration in a model of human skin equivalent transplant." Life Sci. 73:1985-94.

Schechner et al. (2003). "Engraftment of a vascularized human skin equivalent." FASEB J. 17(15):2250-60.

Research has been carried out in humans during the last few years to examine the potential benefits of using EPCs and other bone marrow derived cells to treat myocardial disorders. Recent studies demonstrate that implantation of autologous progenitor cells after Acute Myocardial Infarction appears to limit post-infarction damage. The following clinical trials focus on the studies that assessed the safety and efficacy of bone marrow-derived or blood-derived cells administered in patients with cardiac disorders.

Perin et al. carried out a clinical trial which included 21 patients (14 in the treatment group, 7 in the control group) who received transendocardial injections of autologous mononuclear BMCs. (See Perin, E. C., H. F. Dohmann, et al. (2003), "Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure," Circulation 107(18): 2294-302, which is incorporated herein by reference.) At 4 months, there was an improvement in ejection fraction, a reduction in end-systolic volume, and significant mechanical improvement of the injected segments in the treated patients.

Another group injected autologous EPCs into the infarct border zone in six patients who had suffered from myocardial infarction and undergone coronary artery bypass grafting. Three to nine months after surgery, all patients were alive and well, and global left-ventricular function was enhanced in four patients. All six patients reported a notable improvement in exercise capacity. Myocardial perfusion scans were reported to have improved strikingly by qualitative analysis in five of six patients. The results of this study indicate that implantation of EPCs to the heart probably induces angiogenesis, thus improving perfusion of the infarcted myocardium. (See Stamm, C., B. Westphal, et al. (2003), "Autologous bone-marrow stem-cell transplantation for myocardial regeneration," Lancet 361(9351): 45-6, which is incorporated herein by reference.)

The Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI) study involved the delivery of circulating endothelial progenitor cells or bone marrow cells directly into coronary arteries after the infarction in patients with reperfused acute myocardial infarction (See Assmus, B., V. Schachinger, et al. (2002), "Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI)," Circulation 106(24): 3009-17, which is incorporated herein by reference.) In the first 20 patients, 11 received EPCs and 9 received BMCs. At 4 months, transplantation of progenitor cells resulted in a significant increase in global left ventricular ejection fraction, echocardiography revealed improved regional wall motion in the infarct zone, reduced end-systolic left ventricular volumes, and increased myocardial viability in the infarct zone compared with a nonrandomized, matched reference group. There were no adverse events of treatment in any of the patients such arrhythmias, or increase in creatine kinase and troponin. There was no difference between BM- and peripheral blood-derived cells.

A study performed by a team led by Amit Patel of University of Pittsburgh, involved 20 patients with severe heart failure out of which 10 were injected into the coronary vessels with BM derived EPCs. At one-, three- and six-month follow-up, the ejection fraction rates for the stem cell patients were significantly improved compared to the other patients. (See Abstract from American Association for Thoracic Surgery, Toronto, May 2004).

The following articles, which are incorporated herein by reference, may also be of interest:

J. Folkman, Y. Shing, J. Biol. Chem. 267, 10931 (1992)

W. Brugger, S. Heimfeld, R. J. Berenson, R. Mertelsmann, L. Kanz, N. Engl J. Med. 333, 283 (1995)

F. Katz, R. W. Tindle, D. R. Sutherland, M. D. Greaves, Leuk. Res. 9, 191 (1985)

R. G. Andrews, J. W. Singer, I. D. Bernstein, Blood 67, 842 (1986)

B. I. Terman, M. Dougher-Vermazen, M. E. Carrion, D. Dimitrov, D. C. Armellino, et al, Biochem. Biophys. Res. Commun. 187, 1579 (1992)

B. Millauer, S. Wizigmann-Voos, H. Schnurch, R. Martinez, N. P. H. Moller, et al, Cell 72, 835 (1993)

J. C. Voyta, D. P. Via, C. E. Butterfield, B. R. Zetter, J. Cell Biol. 99, 2034 (1984)

P. J. Newman, M. C. Berndt, J. Gorski, G. C. White, S. Lyman, et al, Science 247, 1219 (1990)

T. N. Sato, Y. Tozawa, U. Deutsch, K. Wolburg-Buchholz, Y. Fujiwara, et al, Nature 376, 70 (1995)

H. Schnurch, W. Risau, Development 119, 957 (1993)

J. L. Liesveld, K. E. Frediani, A. W. Harbol, J. F. DiPersio, C. N. Abboud, Leukemia 8, 2111 (1994).

S. Takeshita, L. P. Zheng, E. Brogi, M. Kearney, L. Q. Pu, et al, J. Clin. Invest. 93, 662 (1994)

R. Baffour, J. Berman, J. L. Garb, S. W. Rhee, J. Kaufman, et al, J. Vasc. Surg. 16, 181 (1992)

J. M. Isner, A. Pieczek, R. Schainfeld, R. Blair, L. Haley, et al, Lancet 348, 370 (1996)

Y. Sato, K. Okamura, A. Morimoto, R. Hamanaka, K. Hamanaguchi, et al, Exp. Cell Res. 204, 223 (1993)

The following articles, which are also incorporated herein by reference, may also be of interest:

Badorff, C., R. P. Brandes, et al. (2003). "Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes." Circulation 107(7): 1024-32.

Bhattacharya, V., P. A. McSweeney, et al. (2000). "Enhanced endothelialization and microvessel formation in polyester grafts seeded with CD34(+) bone marrow cells." Blood 95(2): 581-5.

Grant, M. B., W. S. May, et al. (2002). "Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization." Nat Med 8(6): 607-12.

Hirata, K., T. S. Li, et al. (2003). "Autologous bone marrow cell implantation as therapeutic angiogenesis for ischemic hindlimb in diabetic rat model." Am J Physiol Heart Circ Physiol 284(1): H66-70.

Ikenaga, S., K. Hamano, et al. (2001). "Autologous bone marrow implantation induced angiogenesis and improved deteriorated exercise capacity in a rat ischemic hindlimb model." J Surg Res 96(2): 277-83.

Kalka, C., H. Masuda, et al. (2000). "Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization." Proc Natl Acad Sci USA 97(7): 3422-7.

Kaushal, S., G. E. Amiel, et al. (2001). "Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo." Nat Med 7(9): 1035-40.

Kornowski, R., M. B. Leon, et al. (2000). "Electromagnetic guidance for catheter-based transendocardial injection: a platform for intramyocardial angiogenesis therapy. Results in normal and ischemic porcine models." J Am Coll Cardiol 35(4): 1031-9.

Li, R. K., Z. Q. Jia, et al. (1996). "Cardiomyocyte transplantation improves heart function." Ann Thorac Surg 62(3): 654-60; discussion 660-1.

Rajnoch, C., J. C. Chachques, et al. (2001). "Cellular therapy reverses myocardial dysfunction." J Thorac Cardiovasc Surg 121(5): 871-8.

Schatteman, G. C., H. D. Hanlon, et al. (2000). "Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice." J Clin Invest 106(4): 571-8.

Shintani, S., T. Murohara, et al. (2001). "Augmentation of postnatal neovascularization with autologous bone marrow transplantation." Circulation 103(6): 897-903.

Strauer, B. E., M. Brehm, et al. (2002). "Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans." Circulation 106(15): 1913-8.

Taylor, D. A., B. Z. Atkins, et al. (1998). "Regenerating functional myocardium: improved performance after skeletal myoblast transplantation." Nat Med 4(8): 929-33.

Thompson, C. A., B. A. Nasseri, et al. (2003). "Percutaneous transvenous cellular cardiomyoplasty. A novel nonsurgical approach for myocardial cell transplantation." J Am Coll Cardiol 41(11): 1964-71.

Tomita, S., R. K. Li, et al. (1999). "Autologous transplantation of bone marrow cells improves damaged heart function." Circulation 100(19 Suppl): II247-56.

Tomita, S., D. A. Mickle, et al. (2002). "Improved heart function with myogenesis and angiogenesis after autologous porcine bone marrow stromal cell transplantation." J Thorac Cardiovasc Surg 123(6): 1132-40.

Wang et al. (2004). "Rosiglitazone facilitates angiogenic progenitor cell differentiation toward endothelial lineage: a new paradigm in glitazone pleiotropy." Circulation 109(11): 1392-400.

Rupp et al. (2004). "Statin therapy in patients with coronary artery disease improves the impaired endothelial progenitor cell differentiation into cardiomyogenic cells." Basic Res Cardiol. 99(1): 61-8.

Quirici et al. (2001). "Differentiation and expansion of endothelial cells from human bone marrow CD133(+) cells." Br J Haematol. 115(1): 186-94.

Di Stefano et al. (2002) "Different growth conditions for peripheral blood endothelial progenitors." Cardiovasc Radiat Med. 3(3-4): 172-5.

Akita et al. (2003). "Hypoxic preconditioning augments efficacy of human endothelial progenitor cells for therapeutic neovascularization." Lab Invest. 83(1): 65-73.

Wang et al. (2004). "Mechanical, cellular, and molecular factors interact to modulate circulating endothelial cell progenitors." Am J Physiol Heart Circ Physiol. 286(5): H1985-93.

Bahlmann et al. (2003). "Endothelial progenitor cell proliferation and differentiation is regulated by erythropoietin." Kidney Int. 64(5): 1648-52.

Heeschen et al. (2003). "Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization." Blood. 102(4): 1340-6.

Verma et al. (2004). "C-reactive protein attenuates endothelial progenitor cell survival, differentiation, and function: Further evidence of a mechanistic link between C-reactive protein and cardiovascular disease." Circulation. 109(17): 2058-67.

US Patents 5,980,887, 6,569,428, and 6,676,937 to Isner et al., which are incorporated herein by reference, generally describe pharmaceutical products including EC progenitors for use in methods for regulating angiogenesis, i.e., for enhancing or inhibiting blood vessel formation, in a selected patient and in some preferred embodiments for targeting an angiogenesis modulator to specific locations. For example, the EC progenitors can be used to enhance angiogenesis or to deliver an angiogenesis modulator, e.g., anti- or pro-angiogenic agents, respectively to sites of pathologic or utilitarian angiogenesis. Additionally, in another embodiment, EC progenitors can be used to induce re-endothelialization of an injured blood vessel, and thus reduce restenosis by indirectly inhibiting smooth muscle cell proliferation.

US Patent 5,541,103 to Kanz et al., which is incorporated herein by reference, describes high-dose chemotherapy treatments for patients suffering from certain types of cancer. In order to facilitate recovery, a process for the ex vivo expansion of peripheral blood progenitor cells is described, wherein CD34+ cells are enriched and cultivated in a medium comprising IL-1, IL-3, IL-6, EPO and SCF. The ex vivo expanded peripheral blood progenitor cells can be administered to cancer patients after chemotherapy.

US Patent Application Publication 2003/0199464 to Itescu, which is incorporated herein by reference, describes a method for treating a disorder of a subject's heart

involving loss of cardiomyocytes. The method includes administering to the subject an amount of an agent described as being effective to cause cardiomyocyte proliferation within the subject's heart so as to thereby treat the disorder. In an embodiment, the agent is human endothelial progenitor cells. The application also describes methods for determining the susceptibility of a cardiomyocyte in a subject to apoptosis.

PCT Patent Publication WO 01/94420 to Itescu, which is incorporated herein by reference, describes a method of stimulating vasculogenesis of myocardial infarct damaged tissue in a subject comprising: (a) removing stem cells from a location in the subject; (b) recovering endothelial progenitor cells from the stem cells; (c) introducing the endothelial progenitor cells from step (b) into a different location in the subject such that the precursors migrate to and stimulate revascularization of the tissue. The stem cells may be removed directly or by mobilization. The endothelial progenitor cells may be expanded before introduction into the subject. A method of inducing angiogenesis in peri-infarct tissue is described. A method is also described for selectively increasing the trafficking of human bone marrow-derived endothelial cell precursors to the site of tissue damaged by ischemic injury, which comprises: (a) administering endothelial progenitor cells to a subject; (b) administering chemokines to the subject so as to thereby attract endothelial cell precursors to the ischemic tissue. A method is also described for stimulating vasculogenesis or angiogenesis of myocardial infarct damaged tissue in a subject comprising injecting allogeneic stem cells into a subject. A method is also described for improving myocardial function in a subject that has suffered a myocardial infarct comprising any of the instant methods. A method is also described for improving myocardial function in a subject who has suffered a myocardial infarct comprising injecting G-CSF or anti-CXCR4 antibody into the subject in order to mobilize endothelial progenitor cells.

US Patent 5,199,942 to Gillis, describes a method for autologous hematopoietic cell transplantation of patients receiving cytoreductive therapy, including: (1) obtaining hematopoietic progenitor cells from bone marrow or peripheral blood from a patient prior to cytoreductive therapy; (2) expanding the hematopoietic progenitor cells ex vivo with an ex vivo growth factor selected from the group consisting of interleukin-3 (IL-3), steel factor (SF), granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1 (IL-1), GM-CSF/IL-3 fusion proteins and combinations thereof, to provide

a cellular preparation comprising an expanded population of progenitor cells; and (3) administering the cellular preparation to the patient concurrently with or following cytoreductive therapy. The method optionally includes a preliminary treatment with a recruitment growth factor to recruit hematopoietic progenitor cells into peripheral blood and a subsequent treatment with an engraftment growth factor to facilitate engraftment and proliferation of hematopoietic progenitor cells administered in the cellular preparation. The patent also describes a hematopoietic progenitor cell expansion media composition comprising cell media, an ex vivo growth factor, and autologous serum.

US Patent 4,656,130 to Shoshan, which is incorporated herein by reference, describes a collagen coated cell growth plate that includes a substrate coated with a storage stable coating of collagen fibrils. The method of preparing the collagen coated cell growth plates comprises dispensing biologically active collagen fibrils suspended in distilled water onto a tissue culture dish. Thereafter, the dish containing the collagen fibril suspension is placed in a laminar flow hood provided with a sterile air stream and ultraviolet light. The fibrils sediment and adhere to the bottom of the dish, the water evaporates in the sterile air stream and is removed in the laminar flow hood exhaust, and the ultraviolet light ensures that the resulting thin layer of collagen fibrils is sterile and ready for the inoculation of living cells. The method is described as yielding a convenient precoated cell growth plate which can maintain reasonable shelf life when kept at room temperature without any significant decrease in cell growth support properties.

US Patent 5,932,473 to Swiderek et al., which is incorporated herein by reference, describes a cell culture substrate that is coated with a composition containing a cell adhesion promoter in a salt solution. A substrate such as plastic, glass or microporous fibers is coated with a composition containing about 5 - 1000 ug/ml of poly-D-lysine in an 0.005 - 0.5 M citrate or sulfate salt solution, in order to provide about 50 - 500 ul of the composition per cm2 of substrate. The coated substrate is rinsed to remove extraneous materials, and dried to obtain a coated substrate having increased shelf-life and/or stability. The coated substrate may be sterilized by rinsing with a sterilizing medium such as ethanol.

US Patent 6,040,182 to Septak, which is incorporated herein by reference, describes methods and materials for the facilitation of high-protein-binding capability on tissue culture-treated plastic surfaces, such as, for example, polystyrene assay plates.

US Patent 4,450,231 to Ozkan, which is incorporated herein by reference, describes an immunoassay of a specimen of a serum or the like to determine immune complexes. A method is described which includes producing on a plastic base a layer of a non-proteinaceous, non-ionic polymer which will adhere to the plastic base and has the capability of absorbing immune complexes of the specimen, placing a specimen on the layer and treating the layer to produce an indication of the amount of immune complexes. The polymer may be polyethylene glycol, dextran, polyvinyl chloride, a polymeric polyol or an adduct of polyethylene glycol. A product for use in such an assay is a plate having wells or a test tube formed of plastic, polystyrene and polyvinyl chloride being preferred, with a layer of such non-proteinaceous, non-ionic layer on the plate wells or the cavity of the test tube.

US Patent Application Publication 2003/0229393 to Kutryk et al., which is incorporated herein by reference, describes compositions and methods for producing a medical device such as a stent, a stent graft, a synthetic vascular graft, or heart valves, which are coated with a biocompatible matrix which incorporates antibodies, antibody fragments, or small molecules, which recognize, bind to and/or interact with a progenitor cell surface antigen to immobilize the cells at the surface of the device. The coating on the device can also contain a compound or growth factor for promoting the progenitor endothelial cell to accelerate adherence, growth and differentiation of the bound cells into mature and functional endothelial cells on the surface of the device to prevent intimal hyperplasia. Methods for preparing such medical devices, compositions, and methods for treating a mammal with vascular disease such as restenosis, atherosclerosis or other types of vessel obstructions are described.

SUMMARY OF THE INVENTION

The present patent application details methods for isolation, differentiation and expansion of stem cells from a tissue. For example, the stem cells may include endothelial progenitor cells (EPCs). Alternatively or additionally, the tissue may include human peripheral blood. Typically, the stem cells are transplanted into the donor or into

another individual (e.g., in order to enhance vasculogenesis and/or angiogenesis and/or neovascularization). The present patent application provides protocols for obtaining a product containing appropriate numbers of functional EPCs. The methods described include: (a) Extraction of cellular sub-populations from a tissue; (b) expansion and differentiation of EPCs in culture for 1-30 days (or 3-30 or 4, 5, 6, 7, or 8 days) in enriched culture medium; and/or (c) identification of cellular components of the culture; (d) implantation of appropriate number of EPCs into a patient. It is to be understood that whereas some embodiments described herein relate specifically to EPCs derived from blood, the scope of the present invention includes techniques for use with stem cells derived from a variety of body tissues, *mutatis mutandis*.

For some applications, the method comprises collecting a blood sample from a donor and/or a patient, isolating from the sample peripheral blood mononuclear cells, separating a population of cells rich in CD31, and progenitor cells from the mononuclear cell fraction, and growing these cells under conditions that will cause the hematopoietic progenitor cells present in the mixture of cells to differentiate into EPCs and proliferate. This ex vivo expansion step is typically utilized because the number of EPCs in the circulation is below 0.1%. Following this augmentation stage, the cells may be implanted by injection into blood vessels in the target organ, such as the coronary vessels or into the myocardium of a patient.

There is therefore provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml; and

increasing the number of cells having a density between 1.055 and 1.068 g/ml, by culturing the second-pass cells for a period lasting between 3 and 30 days.

In an embodiment, applying the blood cells to the first gradient includes applying the blood cells to a gradient including copolymers of sucrose and epichlorohydrin such as Ficoll-Paque Plus (TM) (Amersham Biosciences, Uppsala, Sweden) or Lymphoprep (TM) (Axis-Shield PoC AS, Oslo, Norway) or obtainable from another source. In an embodiment, the density gradient is prepared by a technician on site.

In an embodiment, applying the first-pass cells to the second gradient includes applying the first-pass cells to a gradient, including an aqueous solution of Iodixanol such as OptiPrep (TM) or Nycodenz (TM) (Axis-Shield PoC AS, Oslo, Norway).

In an embodiment, applying the first-pass cells to the second gradient includes applying the first-pass cells to a gradient, including polyvinylpyrrolidone-coated silica colloids such as Percoll (TM) (Amersham Biosciences, Uppsala, Sweden).

There is further provided, in accordance with an embodiment of the present invention, a method for use with extracted stem cells, including:

applying tissue including the stem cells to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

applying the second-pass cells to a third gradient suitable for selecting third-pass cells having a density between 1.055 and 1.068 g/ml; and

increasing the number of cells having a density between 1.055 and 1.068 g/ml, by culturing the third-pass cells for a period lasting between 3 and 30 days.

In an embodiment, the third gradient is suitable for selecting cells having a density between 1.059 and 1.068 g/ml, and wherein applying the second-pass cells to the third gradient includes selecting the cells having a density between 1.059 and 1.068 g/ml.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml; and

incubating the second-pass cells on a surface including (e.g., coated with) plasma and/or an antibody.

There is additionally provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml; and

incubating the second-pass cells on a surface including a growth-enhancing molecule other than collagen or fibronectin.

In an embodiment, incubating the second-pass cells includes incubating the second-pass cells on a surface that includes, in addition to the growth-enhancing molecule, at least one of: collagen and fibronectin.

There is yet additionally provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml; and

culturing the second-pass cells for a period lasting between 1 and 5 days in a culture medium including up to 5% serum (e.g., no serum, less than 1% serum, or between 1 and 5% serum).

There is still additionally provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml; and

culturing the second-pass cells for a period lasting between 1 and 5 days in a culture medium including greater than or equal to 10% serum.

In an embodiment, culturing the second-pass cells includes culturing the second-pass cells in a culture medium including less than 20% serum.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

during a low-serum time period, culturing the second-pass cells in a culture medium including less than 10% serum; and

during a high-serum time period, culturing the second-pass cells in a culture medium including greater than 10% serum.

In an embodiment, culturing the second-pass cells during the low-serum time period includes culturing the second-pass cells for a duration of between 1 and 5 days.

In an embodiment, culturing the second-pass cells during the high-serum time period includes culturing the second-pass cells for a duration of between 1 and 30 days.

In an embodiment, culturing the second-pass cells during the low-serum time period is performed prior to culturing the second-pass cells during the high-serum time period.

In an embodiment, culturing the second-pass cells during the low-serum time period is performed following culturing the second-pass cells during the high-serum time period.

There is further provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

during a hypoxic and/or hypercapnic (H/H) time period lasting at least 2 hours, culturing the second-pass cells under H/H conditions; and

during a non-H/H time period lasting at least 1 day, culturing the second-pass cells under non-H/H conditions.

In the context of the present patent application and in the claims, the term hypercapnia refers to a concentration of CO_2 that is greater than 5%.

In an embodiment, the H/H and non-H/H time-periods are within a culturing time period lasting less than 30 days, and culturing the second-pass cells under H/H conditions includes culturing the second-pass cells under H/H conditions during a first two days of the culturing time period.

In an embodiment, the H/H and non-H/H time-periods are within a culturing time period lasting less than 30 days, and culturing the second-pass cells under H/H conditions includes culturing the second-pass cells under H/H conditions during a last two days of the culturing time period.

In an embodiment, the H/H and non-H/H time-periods are within a culturing time period lasting less than 30 days, and culturing the second-pass cells under H/H conditions includes culturing the second-pass cells under H/H conditions for at least 2 hours between a first two days and a last two days of the culturing time period.

In an embodiment, culturing the second-pass cells under H/H conditions is performed prior to culturing the second-pass cells under non-H/H conditions.

In an embodiment, culturing the second-pass cells under H/H conditions is performed following culturing the second-pass cells under non-H/H conditions.

There is still further provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml; and

culturing the second-pass cells in a culture medium including at least one of the following: erythropoietin, VEGF, IGF, FGF, a molecule from the estrogen family (e.g., 17-β-estradiol, estrone, estriol, an estradiol derivative, estradiol valerate, estradiol cypionate, mestranol, quinestrol), a molecule from the progestin-family (e.g., progesterone, hydroxyprogesterone caroate, medroxyprogesterone acetate), a statin (e.g. Simvastatin, Atorvastatin), and an antidiabetic agent (e.g., Rosiglitazone).

In an embodiment, the antidiabetic agent includes Rosiglitazone, and culturing the second-pass cells includes culturing the second-pass cells in a culture medium including Rosiglitazone.

In an embodiment, the statin includes Simvastatin or Atorvastatin, and culturing the second-pass cells includes culturing the second-pass cells in a culture medium including Simvastatin or Atorvastatin.

In an embodiment, hormone molecules from the estrogen and progestin families include 17-β-estradiol and progesterone, and culturing the second-pass cells includes culturing the second-pass cells in a culture medium including 17-β-estradiol and/or progesterone.

In an embodiment, hormone molecules from the estrogen and progestin families include 17-β-estradiol and progesterone, and culturing the second-pass cells includes culturing the second-pass cells in a culture medium including 17-β-estradiol and to which progesterone is added after a certain period.

In an embodiment, hormone molecules from the estrogen and progestin families include 17-β-estradiol and progesterone, and culturing the second-pass cells includes culturing the second-pass cells in a culture medium including progesterone and to which 17-β-estradiol is added after a certain period.

There is yet further provided, in accordance with an embodiment of the present invention, a method for use with extracted stem cells, including:

applying tissue including the stem cells to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml; and

increasing the number of cells having a density between 1.059 and 1.068 g/ml, by culturing the second-pass cells for a period lasting between 3 and 30 days.

In an embodiment, the method includes extracting the stem cells from bone marrow.

In an embodiment, the method includes mobilizing the stem cells from bone marrow to facilitate extraction of the stem cells.

In an embodiment, the method includes extracting the stem cells from blood, umbilical cord blood, an embryo, a fetus or a placenta.

In an embodiment, culturing the second-pass cells includes:

culturing the second-pass cells in a first container during a first portion of the period;

removing at least some of the second-pass cells from the first container at the end of the first portion of the period; and

culturing, in a second container during a second portion of the period, the cells removed from the first container.

In an embodiment, removing the at least some of the second-pass cells includes selecting for removal cells that adhere to a surface of the first container.

In an embodiment, removing the at least some of the second-pass cells includes selecting for removal cells that do not adhere to a surface of the first container.

In an embodiment, the first container includes on a surface thereof a growth-enhancing molecule, and culturing the cells in the first container includes culturing the cells in the first container that includes the growth-enhancing molecule.

In an embodiment, the second container includes on a surface thereof a growth-enhancing molecule, and culturing the cells in the second container includes culturing the cells in the second container that includes the growth-enhancing molecule.

In an embodiment, the growth-enhancing molecule is selected from the list consisting of: plasma (which can be autologous, allogeneic or xenogeneic), collagen, fibronectin, a growth factor, and an antibody to a stem cell surface receptor.

There is therefore provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

increasing the number of cells having a density between 1.055 and 1.074 g/ml, by culturing the second-pass cells for a period lasting between 3 and 30 days; and

identifying endothelial progenitor cells in the cultured cells.

In an embodiment, applying the blood to the first gradient includes applying the blood to a solution including a copolymer of sucrose and epichlorohydrin.

In an embodiment, applying the first-pass cells to the second gradient includes applying the first-pass cells to an aqueous solution of iodixanol.

In an embodiment, applying the first-pass cells to the second gradient includes applying the first-pass cells to a gradual density solution including polyvinylpyrrolidone-coated silica colloids.

In an embodiment, applying the blood cells to the first gradient includes applying the blood cells to a Ficoll-like gradient.

In an embodiment, applying the first-pass cells to the second gradient includes applying the first-pass cells to an OptiPrep-like gradient.

In an embodiment, applying the first-pass cells to the second gradient includes applying the first-pass cells to a Percoll-like gradient.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted stem cells, including:

applying tissue including the stem cells to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

applying the second-pass cells to a third gradient suitable for selecting third-pass cells having a density between 1.055 and 1.068 g/ml;

increasing the number of cells having a density between 1.055 and 1.068 g/ml, by culturing the third-pass cells for a period lasting between 3 and 30 days; and

identifying endothelial progenitor cells in the cultured cells.

In an embodiment, the third gradient is suitable for selecting cells having a density between 1.059 and 1.068 g/ml, and wherein applying the second-pass cells to the third gradient includes selecting the cells having a density between 1.059 and 1.068 g/ml.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

culturing the second-pass cells on a surface including plasma; and

identifying progenitor cells in the cultured cells.

In an embodiment, culturing includes culturing the second-pass cells on the surface, when the surface is coated with autologous plasma.

In an embodiment, culturing includes culturing the second-pass cells on the surface, when the surface is coated with at least one plasma selected from the list consisting of: allogeneic plasma and xenogeneic plasma.

There is also provided, in accordance with an embodiment of the present invention, a method for use with tissue, including:

culturing the tissue on a surface including plasma.

In an embodiment, the tissue includes blood.

In an embodiment, the plasma includes autologous plasma.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

culturing the second-pass cells on a surface including an antibody; and

identifying progenitor cells in the cultured cells.

In an embodiment, the progenitor cells include endothelial progenitor cells (EPCs), and wherein identifying the progenitor cells includes identifying the EPCs.

In an embodiment, culturing includes culturing the second-pass cells on the surface, when the surface is coated with autologous plasma.

In an embodiment, culturing includes culturing the second-pass cells on the surface, when the surface is coated with at least one plasma selected from the list consisting of: allogeneic plasma and xenogeneic plasma.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

culturing the second-pass cells on a surface including a growth-enhancing molecule other than collagen or fibronectin; and

identifying progenitor cells in the cultured cells.

In an embodiment, the progenitor cells include endothelial progenitor cells (EPCs), and wherein identifying the progenitor cells includes identifying the EPCs.

In an embodiment, culturing the second-pass cells includes culturing the second-pass cells on a surface that includes, in addition to the growth-enhancing molecule, at least one of: collagen and fibronectin.

There is also provided, in accordance with an embodiment of the present invention, method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1,077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

culturing the second-pass cells for a period lasting between 1 and 5 days in a culture medium including up to 5% serum; and

identifying progenitor cells in the cultured cells.

In an embodiment, the progenitor cells include endothelial progenitor cells (EPCs), and wherein identifying the progenitor cells includes identifying the EPCs.

There is also provided, in accordance with an embodiment of the present invention, method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

culturing the second-pass cells for a period lasting between 1 and 5 days in a culture medium including greater than 10% serum; and

identifying progenitor cells in the cultured cells.

In an embodiment, culturing the second-pass cells includes culturing the second-pass cells in a culture medium including less than 20% serum.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

during a low-serum time period, culturing the second-pass cells in a culture medium including less than 10% serum;

during a high-serum time period, culturing the second-pass cells in a culture medium including greater than or equal to 10% serum; and

identifying progenitor cells in the cultured cells.

In an embodiment, culturing the second-pass cells during the low-serum time period includes culturing the second-pass cells in a culture medium including up to 5% serum.

In an embodiment, culturing the second-pass cells during the low-serum time period includes culturing the second-pass cells in a serum-free culture medium.

In an embodiment, culturing the second-pass cells during the low-serum time period includes culturing the second-pass cells for a duration of between 1 and 5 days.

In an embodiment, culturing the second-pass cells during the high-serum time period includes culturing the second-pass cells for a duration of between 1 and 30 days.

In an embodiment, culturing the second-pass cells during the low-serum time period is performed prior to culturing the second-pass cells during the high-serum time period.

In an embodiment, culturing the second-pass cells during the low-serum time period is performed following culturing the second-pass cells during the high-serum time period.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

during a hypoxic time period lasting at least 2 hours, culturing the second-pass cells under hypoxic conditions;

during a non-hypoxic time period lasting at least 1 day, culturing the second-pass cells under non-hypoxic conditions; and

identifying progenitor cells in the cultured cells.

In an embodiment, the progenitor cells include endothelial progenitor cells (EPCs), and wherein identifying the progenitor cells includes identifying the EPCs.

In an embodiment, the hypoxic and non-hypoxic time-periods are within a culturing time period lasting less than 30 days, and wherein culturing the second-pass cells under hypoxic conditions includes culturing the second-pass cells under hypoxic conditions during a first two days of the culturing time period.

In an embodiment, the hypoxic and non-hypoxic time-periods are within a culturing time period lasting less than 30 days, and wherein culturing the second-pass cells under hypoxic conditions includes culturing the second-pass cells under hypoxic conditions during a last two days of the culturing time period.

In an embodiment, the hypoxic and non-hypoxic time-periods are within a culturing time period lasting less than 30 days, and wherein culturing the second-pass cells under hypoxic conditions includes culturing the second-pass cells under hypoxic conditions for at least 2 hours between a first two days and a last two days of the culturing time period.

In an embodiment, culturing the second-pass cells under hypoxic conditions is performed prior to culturing the second-pass cells under non-hypoxic conditions.

In an embodiment, culturing the second-pass cells under hypoxic conditions is performed following culturing the second-pass cells under non-hypoxic conditions.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a gradient to select cells having a first desired density range;

increasing the number of cells having a second desired density range by culturing the selected cells in a medium including estrogen and subsequently in a medium including a progestin; and

identifying progenitor cells in the cultured cells.

There is also provided, in accordance with an embodiment of the present invention, method for use with extracted blood, including:

applying blood to a gradient to select cells having a first desired density range;

increasing the number of cells having a second desired density range by culturing the selected cells in a medium including a progestin and subsequently in a medium including estrogen; and

identifying progenitor cells in the cultured cells.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a gradient to select cells having a first desired density range;

increasing the number of cells having a second desired density range by culturing the selected cells in a medium including estrogen and a progestin; and

identifying progenitor cells in the cultured cells.

In an embodiment, the progestin includes progesterone.

In an embodiment, the estrogen includes estradiol.

In an embodiment, culturing includes culturing for a period lasting between 3 and 30 days.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

during a hypercapnic time period lasting at least 2 hours, culturing the second-pass cells under hypercapnic conditions, the hypercapnic time period characterized by a CO_2 level of greater than 5%;

during a non-hypercapnic time period lasting at least 1 day, culturing the second-pass cells under non-hypercapnic conditions, the non-hypercapnic time period characterized by a CO_2 level of less than or equal to 5%; and

identifying progenitor cells in the cultured cells.

In an embodiment, setting the CO_2 level during the hypercapnic time period to be at least 6%.

In an embodiment, the hypercapnic and non-hypercapnic time-periods are within a culturing time period lasting less than 30 days, and wherein culturing the second-pass cells under hypercapnic conditions includes culturing the second-pass cells under hypercapnic conditions during a first two days of the culturing time period.

In an embodiment, the hypercapnic and non-hypercapnic time-periods are within a culturing time period lasting less than 30 days, and wherein culturing the second-pass cells under hypercapnic conditions includes culturing the second-pass cells under hypercapnic conditions during a last two days of the culturing time period.

In an embodiment, the hypercapnic and non-hypercapnic time-periods are within a culturing time period lasting less than 30 days, and wherein culturing the second-pass cells under hypercapnic conditions includes culturing the second-pass cells under hypercapnic conditions for at least 2 hours between a first two days and a last two days of the culturing time period.

In an embodiment, culturing the second-pass cells under hypercapnic conditions is performed prior to culturing the second-pass cells under non-hypercapnic conditions.

In an embodiment, culturing the second-pass cells under hypercapnic conditions is performed following culturing the second-pass cells under non-hypercapnic conditions.

There is also provided, in accordance with an embodiment of the present invention, method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

culturing the second-pass cells in a culture medium including at least one agent selected from the list consisting of: erythropoietin, VEGF, IGF, FGF, estrogen, , 17-β-estradiol, estrone, estriol, an estradiol derivative, estradiol valerate, estradiol cypionate, mestranol, quinestrol, progestin, a molecule from the progestin family, progesterone, synthetic progesterone, hydroxyprogesterone caroate, medroxyprogesterone acetate, a statin, simvastatin, atorvastatin, an anti-diabetic agent, and rosiglitazone; and

identifying progenitor cells in the cultured cells.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted stem cells, including:

applying tissue including the stem cells to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

increasing the number of cells having a density between 1.055 and 1.074 g/ml, by culturing the second-pass cells for a period lasting between 3 and 30 days; and

identifying progenitor cells in the cultured cells.

In an embodiment, applying the tissue includes applying umbilical cord blood to the first gradient.

In an embodiment, applying the tissue includes applying embryonic cells to the first gradient.

In an embodiment, applying the tissue includes applying placental cells to the first gradient.

In an embodiment, applying the tissue includes applying fetal cells to the first gradient.

In an embodiment, extracting the stem cells from bone marrow.

In an embodiment, mobilizing the stem cells from bone marrow to facilitate extraction of the stem cells.

In an embodiment, extracting the stem cells from peripheral blood.

In an embodiment, culturing the second-pass cells includes:

culturing the second-pass cells in a first container during a first portion of the period;

removing at least some of the second-pass cells from the first container at the end of the first portion of the period; and

culturing, in a second container during a second portion of the period, the cells removed from the first container.

In an embodiment, removing the at least some of the second-pass cells includes selecting for removal cells that adhere to a surface of the first container.

In an embodiment, removing the at least some of the second-pass cells includes selecting for removal cells that do not adhere to a surface of the first container.

In an embodiment, the first container includes on a surface thereof a growth-enhancing molecule, and wherein culturing the cells in the first container includes culturing the cells in the first container that includes the growth-enhancing molecule.

In an embodiment, the growth-enhancing molecule is selected from the list consisting of: plasma, collagen, fibronectin, a growth factor and an antibody to a stem cell surface receptor.

In an embodiment, the second container includes on a surface thereof a growth-enhancing molecule, and wherein culturing the cells in the second container includes culturing the cells in the second container that includes the growth-enhancing molecule.

In an embodiment, the growth-enhancing molecule is selected from the list consisting of: plasma, collagen, fibronectin, a growth factor and an antibody to a stem cell surface receptor.

There is also provided, in accordance with an embodiment of the present invention, a method for treating a condition, including applying endothelial progenitor cells (EPCs) to a vicinity of tissue or a space of a subject selected from the list consisting of: tissue of a peripheral nerve of the subject, tissue of a central nervous system nerve of the subject, an optic nerve of the subject, choroid tissue of the subject, retinal tissue of the subject, sub-retinal space of the subject, corneal tissue of a subject, kidney tissue of the subject, tissue of a damaged bone of the subject, tissue of a fractured bone of the subject, inflamed tissue of the subject, infected tissue of the subject, contused tissue of

the subject, damaged, ulcerated or wounded tissue of a skin of, brain tissue of the subject, tissue of a limb of the subject, tissue of a skin graft, and tissue of a reattached severed limb of the subject.

In an embodiment, the method includes generating the EPCs by:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml; and

increasing the number of cells having a density between 1.055 and 1.074 g/ml, by culturing the second-pass cells for a period lasting between 3 and 30 days.

In an embodiment, the method includes generating the EPCs by:

applying tissue including the stem cells to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

applying the second-pass cells to a third gradient suitable for selecting third-pass cells having a density between 1.055 and 1.068 g/ml; and

increasing the number of cells having a density between 1.055 and 1.068 g/ml, by culturing the third-pass cells for a period lasting between 3 and 30 days.

In an embodiment, the method includes generating the EPCs by:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml; and

culturing the second-pass cells on a surface including an antibody.

In an embodiment, the method includes generating the EPCs by:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml; and

culturing the second-pass cells on a surface including a growth-enhancing molecule other than collagen or fibronectin.

In an embodiment, the method includes generating the EPCs by:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml; and

culturing the second-pass cells for a period lasting between 1 and 5 days in a culture medium including up to 5% serum.

In an embodiment, the method includes generating the EPCs by:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml; and

culturing the second-pass cells for a period lasting between 1 and 5 days in a culture medium including greater than 10% serum.

In an embodiment, the method includes generating the EPCs by:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

during a low-serum time period, culturing the second-pass cells in a culture medium including less than 10% serum; and

during a high-serum time period, culturing the second-pass cells in a culture medium including greater than or equal to 10% serum.

In an embodiment, the method includes generating the EPCs by:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

during a hypoxic time period lasting at least 2 hours, culturing the second-pass cells under hypoxic conditions; and

during a non-hypoxic time period lasting at least 1 day, culturing the second-pass cells under non-hypoxic conditions.

In an embodiment, the method includes generating the EPCs by:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

during a hypercapnic time period lasting at least 2 hours, culturing the second-pass cells under hypercapnic conditions, the hypercapnic time period characterized by a CO_2 level of greater than 5%; and

during a non-hypercapnic time period lasting at least 1 day, culturing the second-pass cells under non-hypercapnic conditions, the non-hypercapnic time period characterized by a CO_2 level of less than or equal to 5%.

In an embodiment, the method includes generating the EPCs by:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml; and

culturing the second-pass cells in a culture medium including at least one agent selected from the list consisting of: erythropoietin, estrogen, an estrogen-family molecule, 17-β-estradiol, estrone, estriol, an estradiol derivative, estradiol valerate, estradiol cypionate, mestranol, quinestrol, progestin, a progestin-family molecule, progesterone, synthetic progesterone, hydroxyprogesterone caroate, medroxyprogesterone acetate, a statin, simvastatin, atorvastatin, an antidiabetic agent, and rosiglitazone.

applying tissue including the stem cells to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml; and

increasing the number of cells having a density between 1.055 and 1.074 g/ml, by culturing the second-pass cells for a period lasting between 3 and 30 days.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

increasing the number of cells having a density between 1.055 and 1.074 g/ml, by culturing the second-pass cells for a period lasting between 1 and 30 days; and

identifying progenitor cells in the cultured cells.

In an embodiment, the progenitor cells include endothelial progenitor cells (EPCs), and wherein identifying the progenitor cells includes identifying the EPCs.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

dividing the first-pass cells into respective first and second portions thereof;

applying the first portion of the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

mixing the second portion of the first-pass cells with the cells having a density between 1.055 and 1.074 g/ml;

increasing the number of cells having a density between 1.055 and 1.074 g/ml, by culturing the second-pass cells for a period lasting between 3 and 30 days; and

identifying progenitor cells in the cultured cells.

In an embodiment, dividing the first-pass cells includes setting the first portion to be larger than the second portion.

In an embodiment, dividing the first-pass cells includes setting the first portion to be smaller than the second portion.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a gradient to select cells having a first desired density range;

increasing the number of cells having a second desired density range by culturing the selected cells for a period lasting between 3 and 30 days; and

identifying progenitor cells in the cultured cells.

In an embodiment, applying the blood to the gradient includes applying blood to a gradient suitable for selecting cells having a density less than 1.077 g/ml.

In an embodiment, applying the blood to the gradient includes applying blood to a gradient suitable for selecting cells having a density between 1.055 and 1.074 g/ml.

In an embodiment, increasing the number of cells includes culturing the cells for a period lasting between 4 and 8 days.

In an embodiment, applying the blood to the gradient includes applying the blood to a solution including a copolymer of sucrose and epichlorohydrin.

In an embodiment, applying the blood to the gradient includes applying the blood to a gradual density solution including polyvinylpyrrolidone-coated silica colloids.

In an embodiment, applying the blood to the gradient includes applying the blood to an aqueous solution of iodixanol.

In an embodiment, applying the blood to the gradient includes applying the blood to a Ficoll-like gradient.

In an embodiment, applying the blood to the gradient includes applying the blood to an OptiPrep-like gradient.

In an embodiment, applying the blood to the gradient includes applying the blood to a Percoll-like gradient.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a gradient to select cells having a desired density range;

culturing the selected cells for a period lasting between 3 and 30 days on a surface including autologous plasma; and

identifying progenitor cells in the cultured cells.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a gradient to select cells having a desired density range;

culturing the selected cells on a surface including an antibody; and

identifying progenitor cells in the cultured cells.

In an embodiment, culturing includes culturing the cells on the surface, when the surface is coated with autologous plasma.

In an embodiment, culturing includes culturing the cells on the surface, when the surface is coated with at least one plasma selected from the list consisting of: allogeneic plasma and xenogeneic plasma.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a gradient to select cells having a desired density range;

culturing the selected cells on a surface including a growth-enhancing molecule other than collagen or fibronectin; and

identifying progenitor cells in the cultured cells.

In an embodiment, culturing the cells includes culturing the cells on a surface that includes, in addition to the growth-enhancing molecule, at least one of: collagen and fibronectin.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a gradient to select cells having a desired density range;

culturing the selected cells a period lasting between 1 and 5 days in a culture medium including up to 5% serum; and

identifying progenitor cells in the cultured cells.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a gradient to select cells having a desired density range;

culturing the selected cells for a period lasting between 1 and 5 days in a culture medium including greater than 10% serum; and

identifying progenitor cells in the cultured cells.

In an embodiment, culturing the cells includes culturing the cells in a culture medium including less than 20% serum.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a gradient to select cells having a desired density range;

during a low-serum time period, culturing the selected cells in a culture medium including less than 10% serum;

during a high-serum time period, culturing the selected cells in a culture medium including greater than or equal to 10% serum; and

identifying progenitor cells in the cultured cells.

In an embodiment, culturing the cells during the low-serum time period includes culturing the cells in a culture medium including up to 5% serum.

In an embodiment, culturing the cells during the low-serum time period includes culturing the cells in a serum-free culture medium.

In an embodiment, culturing the cells during the low-serum time period includes culturing the cells for a duration of between 1 and 5 days.

In an embodiment, culturing the cells during the high-serum time period includes culturing the cells for a duration of between 1 and 30 days.

In an embodiment, culturing the cells during the low-serum time period is performed prior to culturing the cells during the high-serum time period.

In an embodiment, culturing the cells during the low-serum time period is performed following culturing the cells during the high-serum time period.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a gradient to select cells having a desired density range;

during a hypoxic time period lasting at least 2 hours, culturing the selected cells under hypoxic conditions;

during a non-hypoxic time period lasting at least 1 day, culturing the selected cells under non-hypoxic conditions; and

identifying progenitor cells in the cultured cells.

In an embodiment, the hypoxic and non-hypoxic time-periods are within a culturing time period lasting less than 30 days, and wherein culturing the cells under hypoxic conditions includes culturing the cells under hypoxic conditions during a first two days of the culturing time period.

In an embodiment, the hypoxic and non-hypoxic time-periods are within a culturing time period lasting less than 30 days, and wherein culturing the cells under hypoxic conditions includes culturing the cells under hypoxic conditions during a last two days of the culturing time period.

In an embodiment, the hypoxic and non-hypoxic time-periods are within a culturing time period lasting less than 30 days, and wherein culturing the cells under hypoxic conditions includes culturing the cells under hypoxic conditions for at least 2 hours between a first two days and a last two days of the culturing time period.

In an embodiment, culturing the cells under hypoxic conditions is performed prior to culturing the cells under non-hypoxic conditions.

In an embodiment, culturing the cells under hypoxic conditions is performed following culturing the cells under non-hypoxic conditions.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a gradient to select cells having a desired density range;

during a hypercapnic time period lasting at least 2 hours, culturing the selected cells under hypercapnic conditions, the hypercapnic time period characterized by a CO_2 level of greater than 5%;

during a non-hypercapnic time period lasting at least 1 day, culturing the selected cells under non-hypercapnic conditions, the non-hypercapnic time period characterized by a CO_2 level of less than or equal to 5%; and

identifying progenitor cells in the cultured cells.

In an embodiment, the method includes setting the CO_2 level during the hypercapnic time period to be at least 6%.

In an embodiment, the hypercapnic and non-hypercapnic time-periods are within a culturing time period lasting less than 30 days, and wherein culturing the cells under hypercapnic conditions includes culturing the cells under hypercapnic conditions during a first two days of the culturing time period.

In an embodiment, the hypercapnic and non-hypercapnic time-periods are within a culturing time period lasting less than 30 days, and wherein culturing the cells under

hypercapnic conditions includes culturing the cells under hypercapnic conditions during a last two days of the culturing time period.

In an embodiment, the hypercapnic and non-hypercapnic time-periods are within a culturing time period lasting less than 30 days, and wherein culturing the cells under hypercapnic conditions includes culturing the cells under hypercapnic conditions for at least 2 hours between a first two days and a last two days of the culturing time period.

In an embodiment, culturing the cells under hypercapnic conditions is performed prior to culturing the cells under non-hypercapnic conditions.

In an embodiment, culturing the cells under hypercapnic conditions is performed following culturing the cells under non-hypercapnic conditions.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted blood, including:

applying blood to a gradient to select cells having a first desired density range;

culturing the selected cells in a culture medium including at least one agent selected from the list consisting of: VEGF, IGF, FGF, erythropoietin, estrogen, an estrogen-family molecule, 17-β-estradiol, estrone, estriol, an estradiol derivative, estradiol valerate, estradiol cypionate, mestranol, quinestrol, progestin, a progestin-family molecule, progesterone, synthetic progesterone, hydroxyprogesterone caroate, medroxyprogesterone acetate, a statin, simvastatin, atorvastatin, an antidiabetic agent, and rosiglitazone; and

identifying progenitor cells in the cultured cells.

In an embodiment, the progenitor cells include endothelial progenitor cells (EPCs), and wherein identifying the progenitor cells includes identifying the EPCs.

In an embodiment, applying the blood to the gradient includes applying blood to a gradient suitable for selecting cells having a density less than 1.077 g/ml.

In an embodiment, applying the blood to the gradient includes applying blood to a gradient suitable for selecting cells having a density between 1.055 and 1.074 g/ml.

There is also provided, in accordance with an embodiment of the present invention, a method for use with extracted stem cells, including:

applying tissue including the stem cells to a gradient to select cells having a first desired density range;

increasing the number of cells having a second desired density range, by culturing the selected cells for a period lasting between 3 and 30 days; and

identifying progenitor cells in the cultured cells.

In an embodiment, applying the tissue includes applying umbilical cord blood to the gradient.

In an embodiment, applying the tissue includes applying embryonic cells to the gradient.

In an embodiment, applying the tissue includes applying fetal cells to the gradient.

In an embodiment, applying the tissue includes applying placental cells to the gradient.

In an embodiment, the method includes extracting the stem cells from bone marrow.

In an embodiment, the method includes mobilizing the stem cells from bone marrow to facilitate extraction of the stem cells.

In an embodiment, the method includes extracting the stem cells from blood.

In an embodiment, the method includes culturing the cells includes:

culturing the cells in a first container during a first portion of the period;

removing at least some of the cells from the first container at the end of the first portion of the period; and

culturing, in a second container during a second portion of the period, the cells removed from the first container.

In an embodiment, the method includes removing the at least some of the cells includes selecting for removal cells that adhere to a surface of the first container.

In an embodiment, removing the at least some of the cells includes selecting for removal cells that do not adhere to a surface of the first container.

In an embodiment, the first container includes on a surface thereof a growth-enhancing molecule, and wherein culturing the cells in the first container includes culturing the cells in the first container that includes the growth-enhancing molecule.

In an embodiment, the growth-enhancing molecule is selected from the list consisting of: plasma, collagen, fibronectin, a growth factor and an antibody to a stem cell surface receptor.

In an embodiment, the second container includes on a surface thereof a growth-enhancing molecule, and wherein culturing the cells in the second container includes culturing the cells in the second container that includes the growth-enhancing molecule.

In an embodiment, the growth-enhancing molecule is selected from the list consisting of: plasma, collagen, fibronectin, a growth factor and an antibody to a stem cell surface receptor.

DETAILED DESCRIPTION OF EMBODIMENTS

In accordance with an embodiment of the present invention, a method is provided for isolating, differentiating, and growing endothelial progenitor cells (EPCs) from human peripheral blood. The EPCs are typically implanted in a patient to induce vasculogenesis and/or angiogenesis and/or neovascularization. Typically, peripheral blood mononuclear cells (PBMCs) separated by a density gradient such as Ficoll are further enriched by one or more other density gradients (such as Percoll, OptiPrep, or Nycodenz), and are then allowed to adhere to tissue culture dishes. Cells are typically grown for 3-30 days in an enriched culture medium. At several time points during the culture period, samples are taken for phenotypic assessment. Expanded cells are collected and saved until implantation into the patient.

A series of protocols are described hereinbelow which may be used separately or in combination, as appropriate, in accordance with embodiments of the present invention. It is to be appreciated that numerical values are provided by way of illustration and not limitation. Typically, but not necessarily, each value shown is an example selected from a range of values that is within 25% of the value shown. Similarly, although certain steps are described with a high level of specificity, a person of ordinary skill in the art will appreciate that other steps may be performed, *mutatis mutandis*.

Protocol for tissue culture dish coating

Suitable containers, such as culture dishes, may be coated with one or a combination of EPC-growth-enhancing molecules. The molecules may comprise antibodies to progenitor cell surface receptors such as CD34, CD133, Tie-2, VEGFR-2 (KDR), CD144 or molecules such as LDL, VEGF, FGF, IGF, or platelet-derived growth factor (PDGF).

Example 1: Coating T-75 flasks with autologous plasma

For 20 T-75 flasks: Prepare on the day of cell preparation

Coating of T-75 Flasks surface with the plasma can be done using autologous plasma removed from the centrifuged tissue sample, or alternatively, with plasma from a different source, such as plasma commercially available from Chemicon of Temecula, CA, US.

Collect plasma from the upper fraction of Ficoll tubes

Fill each flask with 2-5 ml plasma.

Incubate at 37°C for at least 30 min.

Discard plasma.

Wash flask twice in 10ml PBS.

Flasks are ready for use.

Example 2: Coating T-75 flasks with 25µg/ml Fibronectin:

For 20 T-75 flasks: Prepare (a) on the day of cell preparation, or (b) one day before the day of cell preparation.

Prepare 50 ml of 25µg/ml Fibronectin solution in PBS.

Add 250µl Fibronectin 5mg/ml to 50ml PBS

Fill each flask with 2-5ml Fibronectin 25µg/ml.

Incubate at 37°C for at least 30min.

Collect Fibronectin solution.

Fibronectin solution can be re-used if stored in sterile 50 ml tube at 4°C, typically for up to 1 week.

Wash flask twice in PBS.

Keep dry flasks at room temperature.

Dried flasks can be saved for one week at room temperature (RT).

Example 3: Coating T-75 flasks with Fibronectin and 5µg/ml anti-CD34:

For 20 T-75 flasks: Prepare (a) on the day of cell preparation, or (b) one day before the day of cell preparation.

Coat flasks with Fibronectin, as described in Example 1.

Prepare 25 ml of 5µg/ml Anti-CD34 solution in PBS.

Add 125µl anti-CD34 1mg/ml to 25ml PBS.

Fill each flask with 5ml anti-CD34 5µg/ml.

Incubate for 2h at 37°C or over night at 4°C.

Withdraw antibody solution.

Wash flask twice in PBS.

Flasks are ready for use.

Cell Preparation

Mix the blood by gently flipping the blood bag up and down.

Transfer blood from bag into a sterile 500ml bottle.

Load blood onto a Ficoll gradient.

Fill tubes with blood up to 50ml.

Spin the tubes for 20 minutes at 1050g at room temperature with no brake.

Collect most of the plasma from the upper layer in empty 50 ml tubes.

Collect the white blood cell ring (e.g., the PBMC) fraction from every tube.

Transfer each PBMC to a new 50ml tube, pre-filled with 15-20 ml of PBS.

Adjust volume to 30ml per tube using PBS.

Spin tubes for 15 minutes at 580g, at room temperature, and discard the supernatant.

Gently mix cell pellet and re-suspend with 1-5ml PBS.

Combine the contents of every four tubes into one 50 ml tube, and fill that tube up to 50 ml with PBS.

For example:

(1) Prepare OptiPrep gradient of 1.072 g/ml.

For 36 ml OptiPrep gradient of 1.072 g/ml

Prepare gradient of 1.072g/ml by mixing together: (i) 25 ml solution of 0.5% human or bovine serum albumin, 0.8% NaCl, 10 mM Hepes, 1 mM EDTA (buffered to pH 7.4) with (ii) 11 ml mixture of 10 ml OptiPrep solution + 5 ml solution of 0.5% human or bovine serum albumin, 0.8% NaCl, 10 mM Hepes, 1 mM EDTA (buffered to pH 7.4).

Mix together: (a) 10 ml cells derived from the Cell Preparation steps described above, with (b) 10 ml mixture of 10 ml OptiPrep solution + 5 ml solution of 0.5% human or bovine serum albumin, 0.8% NaCl, 10 mM Hepes, 1 mM EDTA (buffered to pH 7.4).

Place on top of the mixture of (a) and (b) 20 ml of the OptiPrep gradient of 1.072 g/ml prepared in steps (i) and (ii), and place on top of this 1.5 ml solution of 0.5% human or bovine serum albumin, 0.8% NaCl, 10 mM Hepes, 1 mM EDTA (buffered to pH 7.4).

Centrifuge 30 minutes at 700g, e.g., at room temperature or 4°C, with no brake.

Collect the isolated cells from the interface between the gradient and solution of 0.5% bovine serum albumin, 0.8% NaCl, 10 mM Hepes, 1 mM EDTA, pH 7.4.

Centrifuge 10 minutes at 395g, at room temperature.

Discard supernatant and re-suspend pellet in 10 ml culture medium.

(2) Prepare Percoll gradient for cell density of 1.060-1.068 g/ml.

For example, for 30ml continuous Percoll gradient preparation mix in 50 ml tube:
13.5 ml Percoll (Amersham)

15.0 ml MEM Spinner modification

1.5 ml 10x Earle's salts solution

Centrifuge 10 min at 14000 x g without brakes in a fixed angle rotor.

Carefully take the gradient out of the rotor. The gradient is ready to use now.

The tube should be able to resist centrifugation at 14000 x g. Apply all the cells on the Percoll gradient.

Centrifuge 30 min at 400 x g without brakes.

Collect enriched PBMCs and transfer into a 50 ml tube.

Fill the tube with PBS, centrifuge 10 min at 300 x g.

Resuspend in 50 ml PBS, centrifuge 10 min at 200 x g.

Resuspend in 50 ml PBS, centrifuge 10 min at 200 x g.

Take a 50µl sample for cell counting.

Serum Preparation

Serum can be obtained directly from the patient's coagulated plasma ("off the clot" serum), or prepared from plasma generated from blood pre-treated with an anticoagulant.

For example, for preparation of serum from blood pre-treated with an anticoagulant:

Take plasma that was collected from the upper fraction of Ficoll tubes (See above description of Cell Preparation).

For each 50ml plasma, add 1.2ml 0.8M $CaCl_2 2H_2O$ or any other chemical/biological clotting inducer such as Calcium Chloride, Thromboplastin, Thrombin agonist peptides or others, in order to catalyze the clotting mechanism.

Incubate for 0.5-4 hrs at 37°C.

Spin coagulated plasma for 10 minutes at 3500g.

Collect the serum in a new tube. Do not allow the clot to mix with the serum.

Use collected serum for medium preparation, or aliquot and save at –20°C until use.

Cell Counting

Pre-fill four 96w plates with 50µl Trypan blue (TB) each.

Make 1:5 dilutions by transfer of 20µl of cells sample to one TB containing well, and mix gently by pipetting up and down.

Load 10µl of diluted cells onto each of the 2 chambers of hemocytometer.

Count clear (viable) and blue (dead) cells that lay in the central 25 squares of the upper and lower chambers.

If fewer than 10 cells are counted, make 1:2 dilution by transfer of 50µl of cells sample to 50µl TB.

If more than 200 cells are counted, make 1:25 dilution by transfer of 20µl of 1:5 suspensions to one TB-containing well, and mix gently by pipetting up and down.

Calculate cell number for each chamber according to the following equation:

No. of viable cells x 10,000 x Dilution factor = No. of viable cells /ml

No. of dead cells x 10,000 x Dilution factor = No. of dead cells /ml

% Dead cells = No. of Dead cells/(No. of Viable cells + No. of Dead cells) x 100

% of dead cells should not typically exceed 30%.

Calculate average cell number.

Record counting results.

Summarize final cell numbers and yield of cells/ml blood.

Medium Preparation

Calculate the volume of needed medium

Prepare culture medium.

Medium should contain 1-20% autologous serum.

Medium can contain the following additives in various concentrations of 0.5pg/ml – 1 ng/ml, or 1 ng/ml - 100μg/ml, for example, EPO (0.01-10 IU/ml), IGF (1-100ng/ml), FGF 10-100ng/ml, VEGF (0.5-20 ng/ml) ; Heparin 5-100 IU/ml; different molecules depends on their weight and the desired molarity can range from pg- μg, or the corresponding molarity: Statin molecules (e.g. simvastatin 5-500 μg/ml), antidiabetic agents (e.g., Rosiglitazone 5-500 μg/ml), and/or steroid hormones such as an estrogen (e.g., 17-β-estradiol (2-2000ng/ml) and a progestin (e.g. progesterone 2-2000ng/ml), and combinations thereof.

It is hypothesized that the steroid reproductive system hormones (e.g., estrogens and progestins) exert their effect by elevating EPC blood levels. Thus, applying such molecules or any combination thereof can increase the yield of the production or the differentiation of the progenitors into EPCs *in vitro,* particularly when cells from peripheral blood are subjected to their effects.

Example for Low % Serum Medium

For 100ml medium add:

2μl of VEGF 100μg/ml (final concentration of 10μg/ml)

100μl of Heparin 5000U/ml (final concentration of 5U/ml)

5ml Autologous Serum

94.9ml serum-free medium

Example 1 for High % Serum Medium

For 100ml medium add:

2μl of VEGF 100μg/ml (final concentration of 10μg/ml)

100μl of Heparin 5000U/ml (final concentration of 5U/ml)

20ml Autologous Serum

79.9ml serum-free medium

Example 2 for High % Serum Medium

For 100ml medium add:

5μl of VEGF 100μg/ml (final concentration of 10μg/ml)

100μl of Heparin 5000U/ml (final concentration of 5U/ml)

4μl of Progesterone 5mg/ml (final concentration of 0.2μg/ml)

10ml Autologous Serum

89.9ml serum-free medium

Example 3 for High % Serum Medium

For 100ml medium add:

5μl of VEGF 100μg/ml (final concentration of 10μg/ml)

100μl of Heparin 5000U/ml (final concentration of 5U/ml)

4μl of 17-β-Estradiol 0.05mg/ml (final concentration of 0.002μg/ml)

10ml Autologous Serum

89.9ml serum-free medium

Example 3 for High % Serum Medium

For 100ml medium add:

5μl of VEGF 100μg/ml (final concentration of 10μg/ml)

100μl of Heparin 5000U/ml (final concentration of 5U/ml)

4μl of Progesterone 0.05mg/ml (final concentration of 0.002μg/ml)

4μl of 17-β-Estradiol 0.005mg/ml (final concentration of 0.0002μg/ml)

10ml Autologous Serum

89.9ml serum-free medium

Example 4 for High % Serum Medium

For 100ml medium add:

2µl of VEGF 100µg/ml (final concentration of 10µg/ml)

100µl of Heparin 5000U/ml (final concentration of 5U/ml)

330µl of Simvastatin 570µM (final concentration of 0.95µM)

10ml Autologous Serum

89.6ml serum-free medium

Culturing

Split cells from the combined cell suspension.

Spin tubes for 15 minutes at 500g, room temperature, discard the supernatant.

Gently mix cell pellet and re-suspend cells to 5-50x10^6/ml.

Seed 1-5x10^6 cells/ml.

Incubate flasks at 37°C, 5% CO_2.

Incubate cells in medium containing low serum levels (e.g., up to 5%). Alternatively or additionally, use high (>10%) serum levels.

In accordance with an embodiment of the present invention, the cells are incubated in low-serum medium prior to being incubated in high-serum medium.

Alternatively, the cells are incubated in low-serum medium following being incubated in high-serum medium.

In accordance with an embodiment, (a) incubation in medium comprising 0.5%-5% serum is carried out before incubation in high-serum medium (>10% serum), and (b) incubation in serum-free medium is carried out (i) before the incubation in the 0.5%-5% serum medium, (ii) between the incubation in the medium comprising 0.5%-5% serum and the incubation in the high-serum medium, and/or (iii) following the incubation in the high-serum medium.

Alternatively, (a) incubation in medium comprising 0.5%-5% serum is carried out after incubation in high-serum medium (>10% serum), and (b) incubation in serum-free medium is carried out (i) following the incubation in the 0.5%-5% serum medium,

(ii) between the incubation in the high-serum medium and the incubation in the medium comprising 0.5%-5% serum, and/or (iii) before the incubation in the high-serum medium.

In an embodiment, techniques described herein with respect to low-serum medium are carried out using serum-free medium.

Increased expansion and differentiation of cells may be obtained by exposure of the cell culture to hypoxia and/or hypercapnia (H/H) for 2-12 hours, 12-24 hours, 24-36 hours, or 36-48 hours. This is done one or more times at different points during cell culturing. (See below for a sample applied hypoxia protocol.)

In the context of the present patent application and in the claims, the term hypercapnia refers to a concentration of CO_2 that is greater than 5%.

After the first three days of culture, the cells are grown in a medium containing high levels of serum (e.g., >10%).

Examinations of culture morphology are performed.

Refreshment of medium is performed every 2-3 days.

For example, when cells are cultured in T-75 Flasks:

1. Collect cells in 50 ml tubes.
2. Fill every flask with 5ml fresh medium.
3. Spin tubes for 10 minutes at 450g, room temperature, discard the supernatant.
4. Gently mix cell pellet and resuspend cells in 5ml fresh medium per flask.
5. Return 5ml of cell suspension to every flask.
6. Sample cells for fluorescence-activated cell sorting (FACS) and/or immunohistological analysis every few days (for example, on days 6, 9, 13, 16, 20, 24 and 30).
7. Follow and record culture morphology whenever culture is treated.
8. Sample culture medium from growth dishes for sterility tests at the beginning and end of the procedure, and every 10 days during the culture period.

9. Collect all cultured cells (See section below, "Collection of cells for FACS staining").

Applied hypoxia and/or hypercapnia

For some applications, increased expansion and differentiation of cells may be obtained by exposure of the cell culture, for 2-48 hours, to hypoxic conditions (e.g., 1-5% or 5-15% oxygen) and/or to hypercapnic conditions (e.g., 6-10% CO_2). This is typically done one or more times, at different points during cell culturing.

For example:

On the first day of culture, incubate T-75 flasks in an oxygen controlled incubator. Set the oxygen pressure at 5% and/or set the CO_2 concentration to greater than 5% (e.g., 6% - 8%, or 8% - 10%), and maintain it at this level for 6 hours. Remove the flasks from the incubator and examine the culture. Take a sample of cells and test viability by Trypan blue exclusion method. Set the oxygen pressure of the incubator at 21% and/or set the CO_2 concentration to 5%. Re-insert the flasks into the incubator and continue incubation for the rest of the period. This procedure can be repeated, for example, once a week during the culture period and/or within 24, 48, or 72 hours before termination of the culture.

In an embodiment, cells are cultured in hypercapnic conditions prior to being cultured in an environment of less than or equal to 5% CO_2. Alternatively or additionally, cells are cultured in hypercapnic conditions following being cultured in an environment of less than or equal to 5% CO_2.

Reseeding of adherent and/or detached and/or floating cells

For example:

For some applications, increased expansion and differentiation of cells may be achieved by re-seeding collected cells on new pre-coated dishes in culture medium.

On the third or fourth day of culture, collect all cultured cells. (See section below, "Collection of cells for FACS staining.") Spin tubes for 10 minutes at 450g, at room temperature. Discard the supernatant. Then, gently mix pellet and re-suspend cells in 10ml fresh medium per flask. Finally, seed suspended cells in new pre-coated T-75 flasks. Continue culturing the cells, and perform all other activities (e.g., medium

refreshment, visual inspection, and/or flow cytometry), as appropriate, as described herein.

This procedure can be performed weekly during the culture period and/or within 24, 48, or 72 hours before termination of the culture.

Cell Preservation

Cells can be kept in a preservation medium or frozen in freezing buffer until use, e.g., implantation into the patient.

Collection of cells for FACS staining

Collect cells in 50ml tubes.

Carefully wash flask surface by pipetting with cold PBS, to detach adherent cells.

Collect washed adherent cells in 50ml tubes.

Add 5ml of cold PBS.

Detach remaining adherent cells using gentle round movements with cell scraper.

Collect the detached cells and add them to the tube.

As appropriate, add 5ml EDTA and incubate at 37°C for 5 minutes. Collect the detached cells and add them to the tube.

Spin tube for 5 minutes at 450g, room temperature. Re-suspend the pellet in 2-5 ml PBS.

Count the cells and record counting results.

Summarize final cell numbers and yields / number of seeded cells for every operation day.

Divide equal volumes of cells to FACS.

FACS Staining

Wash cells with PBS.

Spin tubes for 5 minutes at 450g, 4-8°C.

Totally discard the supernatant by pouring the buffer and absorbing remainder on tissue.

Gently mix cell pellet.

Add staining reagent (according to staining table) and mix cells pellet.

Incubate tubes for 15-30 min on ice water in the dark.

Wash the cells with PBS.

Spin tubes for 5 minutes at 450g, 4-8°C.

Totally discard the supernatant by pouring the buffer and absorbing remainder on tissue.

Gently mix cell pellet.

Add 0.5ml PBS per tube (or less, if tube contains less than $1x10^6$ cells).

If aggregates are visible, transfer cell suspension through 200µm mesh.

Read staining results using FACS machine.

Summarize and record FACS results.

Colony formation test

1. Collect cultured cells. (See section, "Collection of cells for FACS staining.")
2. Suspend 100x10^3 cells in 0.7 ml enriched medium containing 50 ng/ml SCF, 2 IU/ml EPO, 5 ng/ml IL-3 and 25 mg/ml BTI–Endothelial cell growth supplement (ECGS) in M199.
3. To a round bottom tube add the following ingredients and mix gently;
   3.1. Methylcellulose 2% - 1.4 ml
   3.2. FCS – 0.9 ml
   3.3. Cell suspension 0.7ml
4. Seed each mix of 3 ml into two 35 mm Petri dishes (1.5ml in each).
5. Place both 35mm Petri dishes in a 100mm Petri dish containing another 35mm Petri dish pre-filled with ddH2O
6. Incubate in 37°C, 5% CO_2, 97% humidity.

7. Score colonies after 10-14 days, using an inverted microscope

Tube formation test

1. Thaw ECMatrix at 4 C overnight.
2. Add 100 microliters of 10X diluent buffer to 900 microliters of ECMatrix solution in a sterile microfuge tube.
3. Mix gently; do not pipette air into the solution. Keep solution on ice to avoid solidification.
4. Transfer 40 microliters buffered ECMatrix solution to each well of a 96-well tissue culture plate that has been pre-cooled at 4 C over night
5. Incubate at 37 C for at least 1 hour to allow the matrix solution to solidify.
6. Collect cultured cells. (See section, "Collection of cells for FACS staining.")
7. Suspend cells to 0.15x10^6/ml in enriched medium containing 10% Human serum, 25 micrograms/ml BTI–Endothelial cell growth supplement (ECGS) and 5 IU/ml heparin in M199
8. Pippette 150 microliter of cell suspension per well onto the surface of the polymerized ECMatrix.
9. Incubate overnight in 37°C, 5% CO_2, 97% humidity
10. Inspect tube formation under an inverted light microscope at 40X-200X magnification.

Cell Specifications

If the cells are to be transplanted into a human, then the following conditions should typically be met:

(I) Cells should be generally free from any bacterial or viral contamination.

(II) Cells should be morphologically characterized as (a) larger in size than lymphocytes and/or (b) elongated, spindle-shaped or irregular-shaped and/or (c)

granulated or dark nucleated and/or (d) with flagella-like structures or pseudopodia and/or (e) fibroblast-like or polygonal in shape.

(III) Final cell suspension should generally contain at least $1x10^6$ cells expressing one or more of the markers: CD31, and/or CD34, and/or CD133 and/or CD34+CD133, and/or KDR, and/or CD34+KDR, and/or CD144, and/or von Willebrand Factor, and/or SH2 (CD105), and/or SH3, and/or fibronectin, and/or collagen (types I, III and IV), and/or ICAM (type 1 or 2) and/or VCAM1 and/or Vimentin and/or BMP-R IA and/or BMP-RII and/or CD44 and/or integrin b1 and/or aSM-actin and/or MUC18 and/or be positive for the enzymatic reaction Dil-Ac-LDL.

Results obtained in accordance with embodiments of the present invention

Example 1. Two-pass isolation of EPCs was carried out in seven independent experiments using Ficoll (first pass) and OptiPrep (second pass), as described above. Results in Table 1 show enrichment of the percentage of CD34+ cells in the second-pass cells. Enrichment is defined as the percentage of CD34+ cells following the second pass divided by the percentage of CD34+ cells following the first pass using OptiPrep.

Table 1. Enrichment of %CD 34 in the Second-Pass cells

Experiment number	% CD34+ Cells		Enrichment Factor
	First Pass	Second Pass	
1	0.2	0.49	2.5
2	<0.2	0.34	>1.7
3	<0.2	0.69	>3.5
4	<0.2	0.65	>3.3
5	<0.2	0.58	>2.9
6	<0.2	<0.2	-
7	<0.2	0.46	>2.3

Example 1. In a set of separate experiments, the ability of first-pass enriched EPCs to generate tubes was evaluated using a tube-formation test following in-vitro growth on Fibronectin-coated T-75 Flasks in the presence of medium containing high serum levels (>10% autologous serum or non-autologous serum), 1, 2, 10 or 20 ng/ml VEGF, and 5-25 IU/ml Heparin. Typical tube formation images are presented in Figure 1. In these experiments conducted with human blood, using the protocol described hereinabove, images were taken using an inverted microscope (Nikon ECLIPSE TS100) using amplifications of x4 and x20 .

Fig. 1 shows tube formation test from first-pass EPCs enrichment in culture.

Example 2. In a set of separate experiments, enrichment of EPCs from second-pass cells was evaluated following in-vitro growth on Fibronectin-coated T-75 Flasks in the presence of medium containing autologous serum, VEGF, b-FGF, IGF, and Heparin. Flow-cytometry percentage staining results from twenty independent experiments are summarized in the Table for example 2. In these experiments conducted with human blood, using the protocol described hereinabove, FACS staining results of cells cultured in media containing high serum levels (>10%) and 1, 2, 10, or 20 ng/ml VEGF yielded the following changes in staining levels from day 0 to day 13:

Table for example 2 Enrichment of Second-Pass EPCs following thirteen days of culture

Marker	%Stained cells on day 0	%Stained cells on day 13
CD45	85% - 98%	7.0% - 39.0%
CD34	Undetectable (<1%)	up to 17.7%
CD133	Undetectable (<1%)	up to 6.5%
KDR	Undetectable (<0.5%)	up to 7.3%

Example 3. In a set of separate experiments, enrichment of EPCs from first-pass cells was evaluated following in-vitro growth on Fibronectin-coated T-75 Flasks in the presence of medium containing autologous serum, VEGF, and Heparin. Flow-cytometry percentage staining results from independent experiments are summarized in the Table for example 3. These experiments were conducted with human blood, using the protocol described hereinabove. Before incubation the cells exhibited less than 1% CD34. FACS staining results of cells cultured in media containing 5-20% autologous serum (typically 10%); 1, 2, 10, or 20 ng/ml VEGF and 5-25 IU/ml Heparin are summarized. The following surface markers were analyzed: CD45 (a pan-lymphocyte marker), the stem/progenitor cell markers CD34 and CD117, and the EPC/endothelial cell markers CD133, KDR (VEGF-R), CD144, and Dil-Ac-LDL.

Table for example 3. Characterization of first-pass EPCs Cultured on Fibronectin pre-coated flasks in the presence of VEGF

Marker	Average (%)	std. Error	N
CD45	89.36	0.85	83
CD34	11.62	1.11	87
CD117	7.01	1.09	45
CD133	2.85	0.46	41
KDR	1.79	0.38	85
CD144	10.33	1.50	3
DIL-Ac-LDL	7.97	0.42	3

Example 4. In a set of separate experiments, enrichment of EPCs from first-pass cells was evaluated following in-vitro growth on autologous plasma-coated T-75 Flasks in the presence of medium containing autologous serum, VEGF and Heparin. Flow-cytometry percentage staining results from 17 independent experiments are summarized in the Table for example 4. These experiments were conducted with human blood, using the protocol described hereinabove. Before incubation the cells exhibited less than 1% CD34. FACS staining results of cells cultured in media containing 5-20% autologous serum (typically 10%), 1, 2, 10, or 20 ng/ml VEGF and 5-25 IU/ml Heparin are summarized. The following surface markers were analyzed: CD45 (a pan-lymphocyte marker), the stem/progenitor cell markers CD34 and CD117, and the EPC/endothelial cell markers CD133 and KDR (VEGF-R).

Table for example 4. Characterization of first-pass EPCs cultured on plasma-coated flasks

Marker	AVG	SE
CD45	89.46	1.75
CD34	6.94	1.29
CD117	4.25	0.72
CD133	2.03	0.57
KDR	1.31	0.39

Example 5. In a set of separate experiments, enrichment of EPCs from first-pass cells was evaluated following in-vitro growth on autologous plasma-coated T-75 Flasks in the presence of medium containing autologous serum, VEGF, progesterone and Heparin. Flow-cytometry percentage staining results from 3 independent experiments are summarized in the Table for example 5. These experiments were conducted with human blood, using the protocol described hereinabove. Before incubation the cells exhibited less than 1% CD34. FACS staining results of cells cultured in media containing 5-20% autologous serum (typically 10%), 1, 2, 10, or 20 ng/ml VEGF, 0.02-2 microgram/ml progesterone and 5-25 IU/ml Heparin are summarized. The following surface markers were analyzed: CD45 (a pan-lymphocyte marker), the stem/progenitor cell markers CD34 and CD117, and the EPC/endothelial cell markers CD133 and KDR (VEGF-R).

Table for example 5. Characterization of first-pass EPCs cultured on plasma pre-coated flasks in the presence of progesterone

Marker	AVG	SE
CD45	94.82	2.31
CD34	21.22	3.47
CD117	5.54	2.08
CD133	5.72	0.47
KDR	1.05	0.12

Example 6. In a set of separate experiments, enrichment of EPCs from first-pass cells was evaluated following in-vitro growth on autologous plasma-coated T-75 Flasks in the presence of medium containing autologous serum, VEGF, 17-beta-estradiol and Heparin. Flow-cytometry percentage staining results from 3 independent experiments are summarized in the Table for example 6. These were experiments conducted with human blood, using the protocol described hereinabove. Before incubation the cells exhibited less than 1% CD34. FACS staining results of cells cultured in media containing 5-20% autologous serum (typically 10%), 1, 2, 10, or 20 ng/ml VEGF, 0.002-2 microgram/ml 17-β-Estradiol and 5-25 IU/ml Heparin are summarized. The following surface markers were analyzed: CD45 (a pan-lymphocyte marker), the stem/progenitor cell markers CD34 and CD117, and the EPC/endothelial cell markers CD133, and KDR (VEGF-R).

Table for example 6. Characterization of first-pass EPCs cultured on plasma pre-coated flasks in the presence of 17-β-estradiol

Marker	average	SE
CD45	96.14	0.38
CD34	4.12	0.05
CD117	1.41	0.27
CD133	1.77	0.88
KDR	0.28	0.20

Example 7. In a set of separate experiments, enrichment of EPCs from first-pass cells was evaluated following in-vitro growth on T-75 Flasks coated with plasma and anti-CD34 antibodies in the presence of medium containing autologous serum, VEGF and Heparin. Flow-cytometry percentage staining results from 2 independent experiments are presented in the Table for example 7. These were experiments conducted with human blood, using the protocol described hereinabove. Before incubation the cells exhibited less than 1% CD34. FACS staining results of cells cultured in media containing FACS staining results of cells cultured on T-75 flasks coated with 5 ml autologous plasma and 0.5-10 micrograms/ml anti-human CD34 in media containing 5-20% autologous serum (typically 10%), 1, 2, 10, or 20 ng/ml VEGF and 5-25 IU/ml Heparin are presented. The following surface markers were analyzed: CD45 (a pan-lymphocyte marker), the stem/progenitor cell markers CD34 and CD117, and the EPC/endothelial cell markers CD133 and KDR (VEGF-R).

Table for example 7. Characterization of first-pass EPCs cultured on plasma and anti-CD34 coated flasks

Marker	Exp. #1	Exp. #2
CD45	85.34	97.10
CD34	1.96	1.50
CD117	0.64	0.83
CD133	0.57	3.88
KDR	0.24	0.54

Example 8. In a set of separate experiments, enrichment of EPCs from first-pass cells was evaluated following in-vitro growth on Fibronectin-coated T-75 Flasks in the presence of medium containing autologous serum, VEGF and Heparin in a high $\%CO_2$ humidified environment. Flow-cytometry percentage staining results from 3 independent experiments are summarized in the Table for example 8. These experiments were conducted with human blood, using the protocol described hereinabove. Before incubation the cells exhibited less than 1% CD34. 5-20% autologous serum (typically 10%), 1, 2, 10, or 20 ng/ml VEGF, 0.002-2 microgram/ml 17-β-Estradiol and 5-25 IU/ml Heparin that were incubated in 37°C, 6.5 – 12.5 % CO_2 and 97% humidity are presented. The following surface markers were analyzed: CD45 (a pan-lymphocyte marker), the stem/progenitor cell marker CD34, and the EPC/endothelial cell marker CD133.

Table for example 8. Characterization of first-pass EPCs cultured in a humid environment containing hypercapnic conditions

Marker	AVG	SE
CD45	90.47	4.53
CD34	23.77	17.93
CD133	4.48	2.10

Complete or partial loss of blood supply to body tissues (ischemia) is a common mechanism in many diseases either as a cause or as an intermediate stage responsible for the disease's outcome. This deficit in supply leads to the affected tissue becoming progressively incapable of performing its functions, to the onset of pathologic processes resulting from the deprivation of nutrients, mainly oxygen, as well as accumulation of metabolites, such as CO_2. If these processes are severe, they eventually lead to cell and tissue death.

The induction of new blood vessel formation to augment or replace the compromised blood supply leads to restoration of function of the affected tissue or organ, and prevention of death of the affected cells. The principle of restoring blood supply to a deprived organ is practiced by cardiologists (e.g., by coronary vessel graft surgery and balloon angioplasty) in order to restore the functioning of the heart and preventing further deterioration.

This invasive approach is generally possible only when large and medium sized vessels are occluded. However, in many diseases associated with a decrease in vascularization, the occlusion occurs in small vessels not amenable to this type of intervention.

In an embodiment of the present invention, EPCs are supplied to a blood-deprived organ or tissue in order to augment or replace the defective vascularization by creating new blood vessels in the deprived organ using autologous or non-autologous endothelial progenitor/stem cells injected either directly into the ischemic tissue, or into patent blood vessels in the proximity of the ischemic tissue. A successful creation of such vasculature generally restores the function, prevents further deterioration, forestalls the formation of pathological processes secondary to blood supply deprivation, and/or averts death of cells in the affected organ. Furthermore, in some cases, controlled blood vessel formation in certain regions of the organ reduce pathological vascularization of other regions within the organ that may reduce the organ's function.

In an embodiment of the present invention, supplying EPCs to an organ or tissue treats one or more of the following ischemia-related conditions. The resulting amelioration of the compromised blood supply generally partially or completely cures the disease or leads to cessation or slowing down of the progression of the condition.

- Glaucoma. This disease consists of progressive death of optic nerve fibers associated with ischemia of the optic nerve head. A treatment leading to restoration of the optic nerve head vasculature generally arrests the disease process and restores some of the function of the optic nerve (at least in those optic nerve fibers whose cell bodies have not yet died, in spite of their axons being compromised at the nerve head). In an embodiment of the present invention, injecting EPCs into the optic nerve head and/or around it generally effects the desired vasculature induction. (See the above-mentioned article by Flammera J et al.)

- Age-related macular degeneration. This disease is associated with circulatory disturbances in the choroid, the blood vessel tissue supplying the outer layers of the retina with its metabolic requirements. These disturbances compromise the retina, leading to its progressive death with consequent reduction in visual functions. In an embodiment of the present invention, injecting EPCs into the choroid generally arrests the disease process and prevents blindness. (See the above-mentioned article by Zarbin MA.)

- Diabetic retinopathy. This disease is a small vessel disease that leads to local ischemia in the retina and therefore edema impairing vision. Although neovascularization occurs, it occurs as a result of ischemia, and it occurs in the region most important to accurate vision – the macula, thereby impairing vision. In an embodiment of the present invention, controlled induction of blood vessel growth by administration of EPCs to regions adjacent to, but not in, the macula reduces or eliminates vessel growth and edema formation in the macular area. This EPC-induced controlled induction of blood vessel growth typically supplies sufficient blood to the retina to obviate the retina's need to vascularize the macular area. (See the above-referenced article by Frank RN, and the above-referenced article by Singleton JR et al.)

- Diabetic nephropathy. This disease involves atherosclerotic blockage of the kidney's blood vessels and destruction of the blood-filtering structures of the kidneys and thus kidney failure, which necessitates dialysis. In an

embodiment of the present invention, induction of replacement vessels by injecting EPCs into the kidneys arrests the process. (See the above-referenced article by Bahlmann FH et al. (2004))

- Non-union of bones. This occurrence after trauma and surgery usually results from insufficient blood supply in the fracture/surgical incision area of the affected bone. In an embodiment of the present invention, local application of EPCs to the fracture/surgical incision area of affected bone restores vascularization and enables healing of the lesion.

- Chronic skin ulcers. These lesions are a result of compromised blood supply to the relevant area of the skin. In an embodiment of the present invention, local application of EPCs to chronic skin ulcers restores the blood supply and generally leads to healing of the wound.

- Vascular dementia, post stroke. This condition results from progressive irreversible closure of blood vessels in the brain. In an embodiment of the present invention, supplying the brain with EPCs restores at least a portion of the compromised circulation and thus restores brain function and/or slows down deterioration of brain function.

- Diabetic vasculopathy. This progressive occlusion of small vessels in the extremities is a common cause for surgical amputations. In an embodiment of the present invention, such amputations are prevented by restoring circulation in the affected limbs by local injection of EPCs.

Like all transplants, skin grafts depend on blood supply for survival (see, for example, the above-mentioned book edited by Greenfield, and the above-mentioned article by Kouwenhoven EA et al.). Both free grafts and skin flaps can fail because of inadequate vascularization. Free grafts require adequate vascularization in the bed, and flaps need the continuation of their own blood supply until local anastomoses can be established (see, for example, the above-mentioned articles by Browne EZ et al., Chen et al., and Beatrice et al.). This is also true for transplants made of artificial skin (see, for example, the above-mentioned article by Ferretti et al.). In these cases, good vascularization is a precondition for optimal reinnervation of the graft.

In an embodiment of the present invention, vascularization is induced by seeding skin grafts with EPCs. Such EPC-induced vascularization generally increases the likelihood of skin graft survival. For some applications, this EPC seeding technique is used in combination with techniques for endothelial cells transplantation described in the above-mentioned article by Schechner et al., *mutatis mutandis*.

In an embodiment of the present invention, vascularization is induced by seeding an attachment site with EPCs during reattachment of a severed limb. Such EPC seeding generally helps restore microcirculation to the reattached limb.

It is to be noted that the indications described hereinabove are only examples of the therapeutic uses of EPCs. In other embodiments of the present invention, other conditions in which blood circulation is compromised are treated by appropriate application of EPCs to locations which have insufficient or no blood vessels.

It is also noted that techniques described herein with respect to increasing stem cell populations prior to administration to heart patients may also be adapted for use with patients having any of the conditions described hereinabove.

An embodiment of the present invention comprises practicing a technique described in one or both of the following provisional patent applications (optionally in combination with a technique described herein):

(a) US Provisional Patent Application 60/576,266, filed June 1, 2004, entitled, "In vitro techniques for use with stem cells," and

(b) US Provisional Patent Application 60/588,520, filed July 15, 2004, entitled, "Indications for stem cell use."

Both of these applications are assigned to the assignee of the present patent application, and are incorporated herein by reference.

Safety and efficacy of EPCs enriched from first-pass cells were evaluated in an ongoing clinical study. These experiments were conducted with patients' autologous blood, using the protocol described hereinabove.

Sixteen patients on maximal drug therapy suffering from severe angina pectoris have been enrolled in the clinical trial that was initiated by TheraVitae and performed in accordance with an embodiment of the present invention. The patients are followed for up to 6 months, as is normally done in cardiovascular clinical trials. Each patient

constitutes his/her own control, with post-treatment condition being compared to the pre-treatment status. Some of the results of the first ten patients that have been followed for at least three months are presented below. It should be emphasized that the results presented herein are not complete and a thorough analysis of the data will be performed on completion of the clinical trial.

Safety – The treatment shows a high safety profile. A limited number of patients developed minor adverse events which were defined as possibly related to the therapy (elevated erythrocyte sedimentation rate, chest pain during angiography). These findings disappeared after a short period without affecting the patient's clinical condition.

Efficacy (see table 1, 2 and 3) – The general clinical condition of all of the patients improved, as shown by the improvement in the Canadian Cardiovascular Society Grading Scale for Angina Pectoris (CCS), showing better capacity to exercise. All patients improved their walking ability free of cardiac symptoms. This finding is also supported by the increase in estimated workload, an objective assessment of exercise capacity done by sestamibi scan. Both results show high statistical significance.

Table I: Patient clinical improvement as demonstrated by the Canadian Cardiovascular Society Grading Scale for Angina Pectoris (CCS).

	Mean value before treatment	Mean value after treatment	Mean percent improvement
CCS score	1.92	1. 00	52%
P value	0.0029		

Table II: Patient clinical improvement at 3 months, as demonstrated by 6-minute walk test

	Absolute Values	Percent
Total number of patients that improved	10	100%
Improvement - average (meters)	84	25%
P value	0.0003	

Table III: Patient improvement at 3 months, as demonstrated by the estimated workload (METs)

	Absolute Values	Percent
Number of patients that improved	7	78%
Improvement - average	1.42	20%
P value	0.02	

In an embodiment, EPCs produced by any of the techniques recited in any of the claims hereinbelow are administered to a human or animal.

In an embodiment, EPCs produced by any of the techniques recited in any of the claims hereinbelow are used in the treatment of a vessel and/or heart disorder, or to treat aging, or to treat a systemic disorder, or to treat a multi-system disorder.

For some applications, techniques described herein are applied to animal tissue.

It will be appreciated by persons skilled in the art that the present invention is not limited to what has been particularly shown and described hereinabove. Rather, the scope of the present invention includes both combinations and subcombinations of the various features described hereinabove, as well as variations and modifications thereof that are not in the prior art, which would occur to persons skilled in the art upon reading the foregoing description.

CLAIMS

1. A method for use with extracted blood, comprising:

applying blood to a first gradient suitable for selecting first-pass cells having a density less than 1.077 g/ml;

applying the first-pass cells to a second gradient suitable for selecting second-pass cells having a density between 1.055 and 1.074 g/ml;

increasing the number of cells having a density between 1.055 and 1.074 g/ml by culturing the second-pass cells for a period lasting between 1 and 30 days in a culture medium comprising one or more agents selected from the group consisting of: autologous serum, VEGF, b-FGF, IGF and heparin; to provide a cultured cell population enriched in hematopoietic progenitor cells and in particular in endothelial progenitor cells (EPCs).

2. The method according to claim 1 further comprising identifying the EPCs in the cultured cell population.

3. The method according to claim 1 wherein the second-pass cells are cultured for a period lasting between 3 and 30 days.

4. The method according to claim 1, wherein the first gradient comprises a solution including a copolymer of sucrose and epichlorohydrin.

5. The method according to claim 1, wherein the second gradient comprises an aqueous solution of iodixanol.

6. The method according to claim 1, wherein the second gradient comprises a gradual density solution including polyvinylpyrrolidone-coated silica colloids.

7. The method according to claim 1, wherein the first gradient comprises a Ficoll-like gradient.

8. The method according to claim 1, wherein the second gradient comprises a Percoll-like gradient.