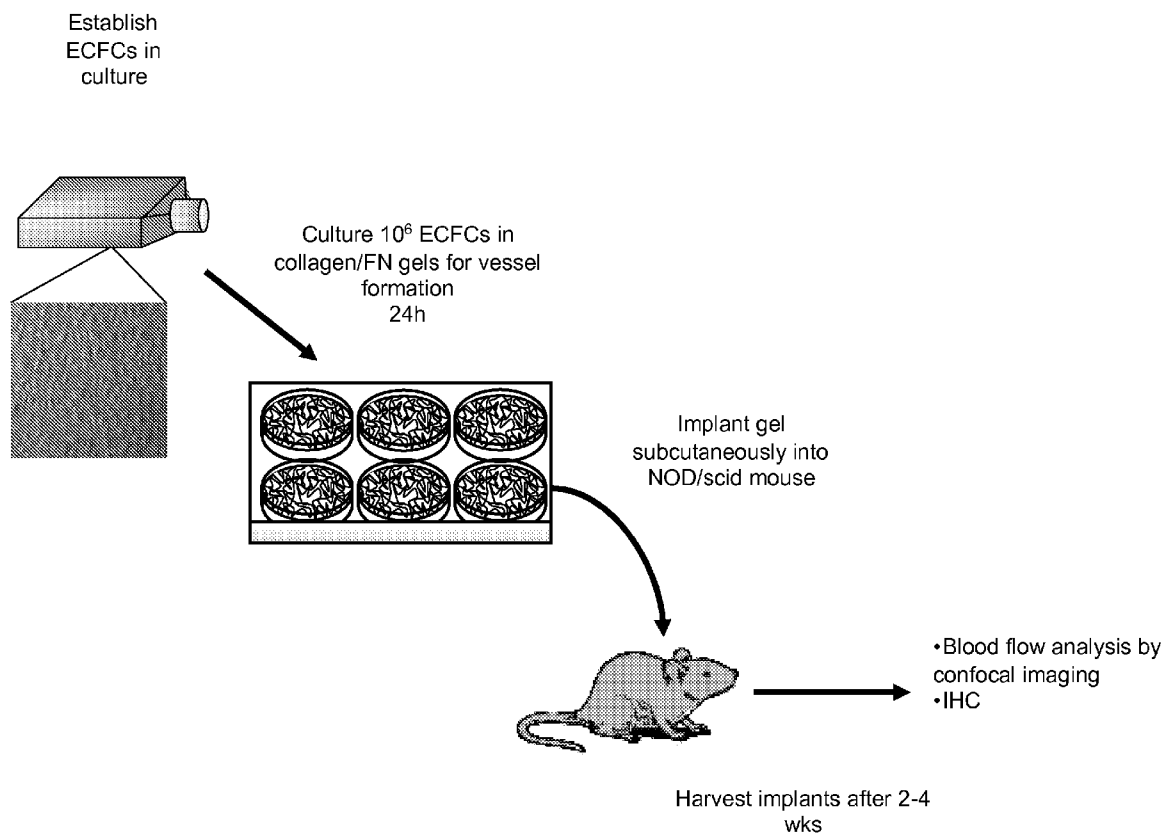


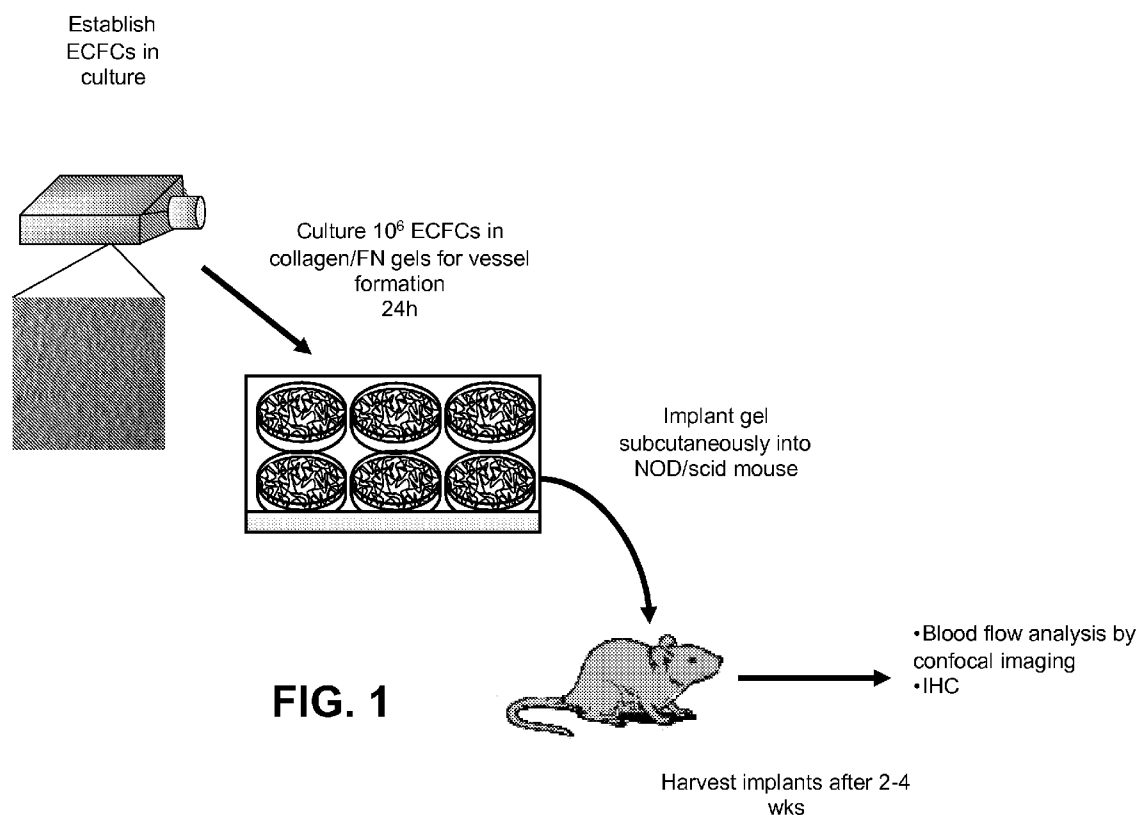


US 20080025956A1

(19) **United States**(12) **Patent Application Publication****Yoder et al.**(10) **Pub. No.: US 2008/0025956 A1**(43) **Pub. Date: Jan. 31, 2008**(54) **BLOOD VESSEL FORMATION FROM
ENDOTHELIAL COLONY FORMING CELLS**(75) Inventors: **Mervin C. Yoder**, Indianapolis, IN
(US); **David A. Ingram**, Indianapolis,
IN (US); **Daniel Prater**, Fishers, IN
(US)Correspondence Address:
BARNES & THORNBURG LLP
11 SOUTH MERIDIAN
INDIANAPOLIS, IN 46204 (US)(73) Assignee: **Indiana University Research and Tech-
nology Corporation**, Indianapolis, IN(21) Appl. No.: **11/837,999**(22) Filed: **Aug. 13, 2007****Related U.S. Application Data**(63) Continuation-in-part of application No. 11/055,182,
filed on Feb. 9, 2005.(60) Provisional application No. 60/822,166, filed on Aug.
11, 2006. Provisional application No. 60/543,114,
filed on Feb. 9, 2004. Provisional application No.
60/542,949, filed on Feb. 9, 2004. Provisional appli-
cation No. 60/573,052, filed on May 21, 2004. Provi-
sional application No. 60/637,095, filed on Dec. 17,
2004.**Publication Classification**(51) **Int. Cl.****A61K 35/12** (2006.01)**A61P 9/00** (2006.01)**C12N 5/02** (2006.01)**C12N 5/08** (2006.01)(52) **U.S. Cl.** **424/93.7; 435/325; 435/402**

(57)

ABSTRACTMethods and compositions to form fully functional blood
vessels in vivo using endothelial colony forming cells
(ECFCs) are disclosed. Culturing ECFCs in a support mate-
rial results in association of ECFCs in vitro and formation of
blood vessels in vivo upon implantation. Direct administra-
tion of cultured ECFCs form blood vessels in vivo. Forma-
tion of blood vessels is useful in treating a variety of medical
conditions including ischemia and hypoxia.



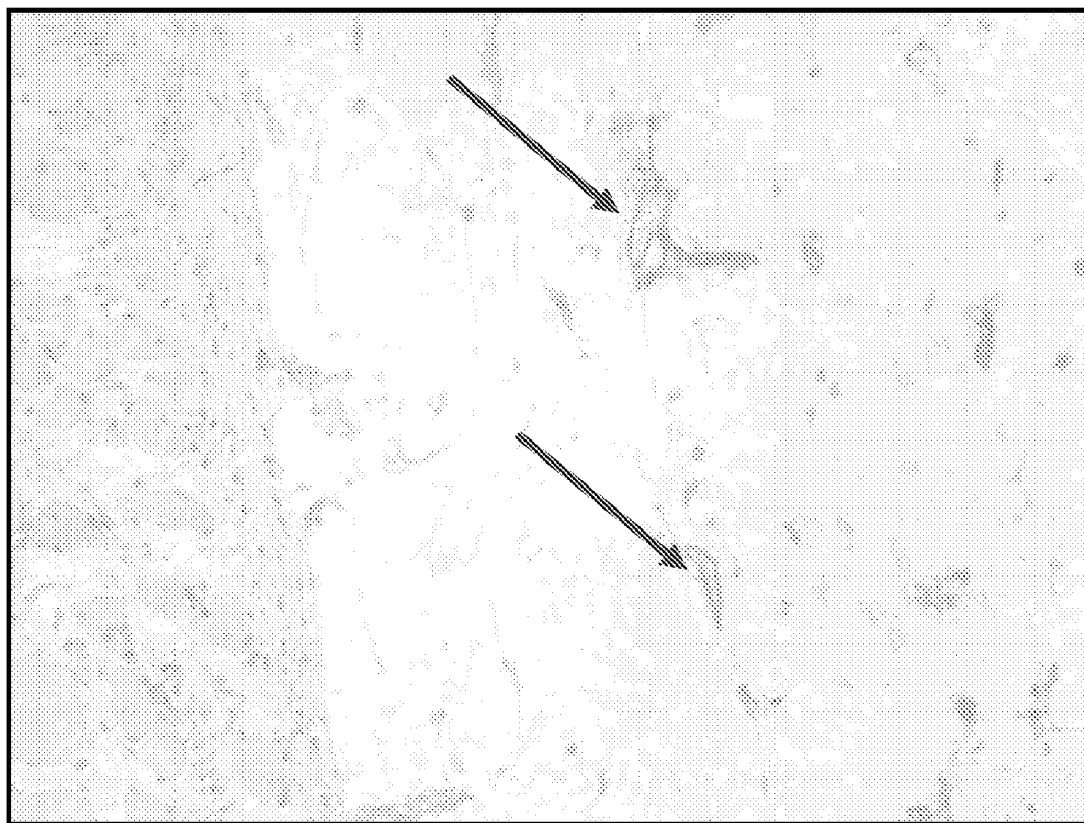


FIG. 2A

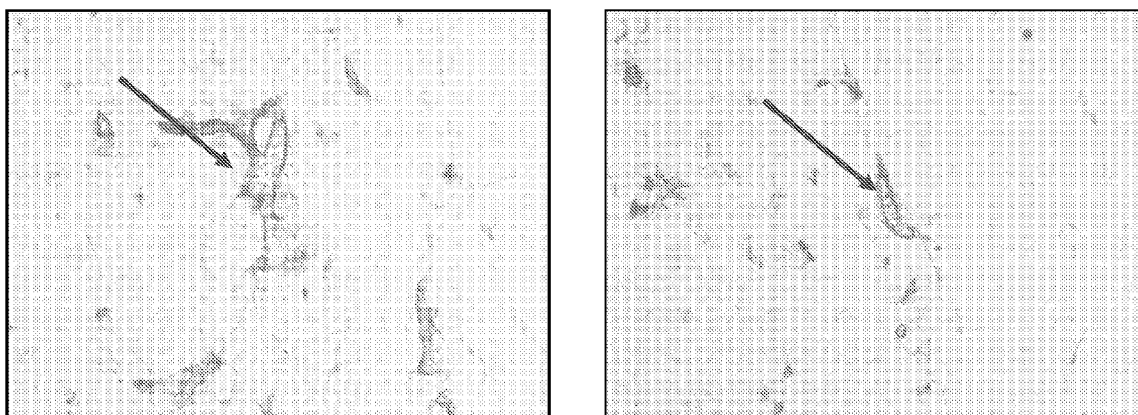


FIG. 2B

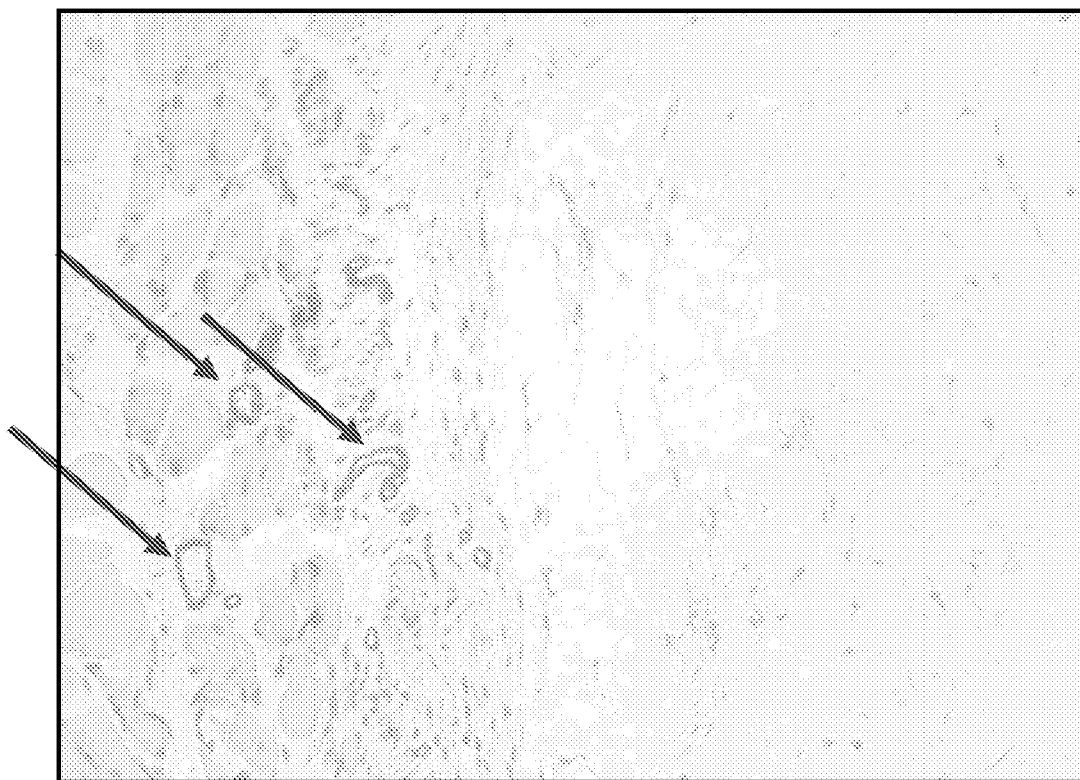


FIG. 3

BLOOD VESSEL FORMATION FROM ENDOTHELIAL COLONY FORMING CELLS

[0001] This application claim priority to U.S. Ser. No. 60/822,166 filed Aug. 11, 2006 and is a continuation-in-part of U.S. Ser. No. 11/055,182 filed Feb. 9, 2005, which claims priority to U.S. Ser. No. 60/543,114 filed Feb. 9, 2004, U.S. Ser. No. 60/542,949 filed Feb. 9, 2004, U.S. Ser. No. 60/573,052 filed May 21, 2004 and U.S. Ser. No. 60/637,095 filed Dec. 17, 2004.

BACKGROUND

[0002] Cord blood circulating endothelial colony forming cells are used to form functional blood vessels in vivo. Methods reproducibly generate human vessels from the cord blood circulating colony forming cells upon implantation into a desired target site.

[0003] During embryogenesis, blood vessels are formed de novo by the patterned assembly of angioblasts in a process termed vasculogenesis. Once an intact vascular system has been established, the development of new blood vessels occurs via the sprouting of endothelial cells from postcapillary venules or the maturation and de novo growth of collateral conduits from larger diameter arteries. These two mechanisms of new blood vessel formation are termed angiogenesis and arteriogenesis, respectively. A population of human circulating CD34+ cells that could differentiate ex vivo into cells with endothelial cell-like characteristics have been termed "endothelial progenitor cells" (EPCs) and may also contribute to new vessel formation. Efforts are focused on defining the role of EPCs in the repair of damaged vascular endothelium or in tumor angiogenesis and on translating these experimental observations into human clinical trials for repair of vascular injury and/or ischemic tissue or as a novel strategy for anticancer therapy.

[0004] New vessel formation occurs via vasculogenesis, angiogenesis, or arteriogenesis. Blood post-natal vasculogenesis has been purported to be an important mechanism for angiogenesis via marrow derived circulating endothelial progenitor cells (EPCs).

[0005] Angiogenesis (neovascularization) is the process of new vessel formation from pre-existing vessels; this is the process reported to give rise to new vessels in adult subjects. Recent studies indicate that marrow-derived EPCs may play minimal or no role in neovascularization of tumors, vessel repair, or normal vessel growth and development. These conflicting reports have raised questions about the function of EPCs in vascular homeostasis and repair. The controversies surrounding these fundamental questions may in part originate from the heterogeneous phenotypic definitions of EPCs and a lack of functional clonogenic assays to isolate and accurately describe the proliferative potential of EPCs. However, there is no uniform definition of an EPC, which makes interpretation of these studies problematic and prohibits reproduction of cell types suitable for clinical use. Although a hallmark of stem and progenitor cells (e.g. hematopoietic, intestinal, neuronal) is their ability to proliferate and give rise to functional progeny, EPCs are primarily defined by the expression of selected cell surface antigens. Sole dependence on cell surface expression of molecules can be problematic because the expression may vary with the physiologic state of the cell. A hallmark of many stem or

progenitor cells in various tissues is their ability to give rise to numerous differentiated progeny to provide sufficient cells for tissue homeostasis.

[0006] Models for stem cell differentiation leading to endothelial and hematopoietic cells are of interest because of the clinical value of stem cells and their progeny. Methods previously used do not guarantee that single endothelial cells have been isolated and characterized to identify the progenitors. Endothelial cell proliferation in vivo in normal, mature arterial, venous, and capillary vessels in most mammals is reported to be extremely low, if not nonexistent.

[0007] Both hematopoietic stem and progenitor cells (HSC/Ps) are enriched in umbilical cord compared to adult peripheral blood. Cord blood is currently used as an alternative resource of hematopoietic stem cells for transplantation of patients with a variety of hematological disorders and malignancies.

[0008] Thousands of patients require a hematopoietic stem cell (HSC) transplant each year. Nearly 2/3 of the patients are unable to find a human leukocyte antigen (HLA) compatible match for the transplant. This is particularly true for many ethnic populations and under-represented minorities. Only 1/3 of Caucasian patients find suitable matched sibling grafts—the most compatible source with the least graft versus host disease (GVHD) complications.

[0009] Human umbilical cord blood is known to be an alternative source of HSCs for clinical transplantation. Whether or not the donor cord blood is a full major histocompatible match to the recipient or is mismatched, cord blood cells engraft and repopulate conditioned hosts as a treatment for a variety of congenital or acquired hematologic disorders. Even if the cord blood graft is mismatched with the recipient by two or more loci, the incidence and severity of GVHD is significantly less than that observed for transplantation of a similarly mismatched adult marrow or mobilized peripheral blood graft.

[0010] Limitations to a more widespread use of cord blood for transplant include the fact that only a limited number of HSC and progenitor cells are present in a graft. Because most patients do not have a matched sibling donor, most cord blood grafts are transplanted into mismatched recipients. Multiple studies report that the dose of cord blood cells in a graft may be a factor for patient survival when the graft comes from an unrelated donor. Transplant related mortality is reported as 20% in recipients that obtained a cord blood graft with more than 1.7×10^5 CD34+ cells/kg versus 75% in those receiving fewer CD34+ cells in the graft. Finding a method to effectively expand cord blood HSC ex vivo to increase the number of cells in a graft, would be a major advance for clinical transplantation and would have a significant commercial market.

SUMMARY

[0011] Method and compositions using endothelial colony forming cells to provide vasculature are disclosed. For example, methods to suspend human cord blood circulating endothelial colony forming cells in collagen-fibronectin gels to form functional blood vessels in vivo are disclosed. In this embodiment, for example, the suspended cells self-associate in about 12-24 hours in an incubator and are implanted in vivo. The implanted cells autonomously form vascular

structures that connect to nearby blood vessels in the host and carry blood cells, thus indicating that the newly formed vessels are functional.

[0012] Endothelial colony forming cells (ECFCs) participate in vascularization. Cultured ECFCs participate in endothelial network formation (capillary formation) and transplanted ECFCs are incorporated into sites of neovascularization in vivo. For example, transplanted human ECFCs formed capillaries. Transplantation of ECFCs functionally augment neovascularization in response to ischemia, e.g., hindlimb ischemia.

[0013] Cord blood colony forming cells are used to form vessels in human subjects with diminished vessel forming ability and critical limb ischemia.

[0014] Single-cell colony assays were developed to describe novel hierarchy among mammalian endothelial progenitor cells (EPCs) isolated from peripheral blood and umbilical cord and from endothelial cells isolated from umbilical or adult blood vessels. A distinct population of progenitor cells from human, bovine, porcine and rat biological samples was identified based on clonogenic and proliferative potential.

[0015] Endothelial progenitor cells (EPCs) were isolated from adult peripheral and umbilical cord blood and expanded exponentially ex vivo. In contrast, human umbilical vein endothelial cells (HUVECs) or human aortic endothelial cells (HAECs) derived from vessel walls are widely considered to be differentiated, mature endothelial cells (ECs) and are utilized as "controls" for EPC studies. However, similar to adult and cord blood derived EPCs, HUVECs and HAECs derived from vessel walls can be passaged for at least 40 population doublings in vitro. Diversity of EPCs exists in human vessels and provides a conceptual framework for determining both the origin and function of EPCs in maintaining vessel integrity. EPCs are therefore readily obtained for clinical use e.g. grafts, either from peripheral blood or from biopsies of human vessels.

[0016] A method for forming a functional vasculature in vivo in a host, the method includes the steps of:

[0017] (a) culturing a plurality of isolated high proliferative-potential endothelial colony forming cells (HPP-ECFC) in a support material;

[0018] (b) implanting the support material comprising the endothelial colony forming cells to a target site; and

[0019] (c) forming the functional vasculature in vivo in the target site in the host.

[0020] A support material may be an artificial matrix and the matrix may be a gel that includes one or more components selected from the group consisting of collagen, fibronectin, gelatin, laminin and any extracellular matrix constituent.

[0021] In an embodiment, the ECFCs are circulating ECFCs. For example, target site comprises an ischemic injury and may be selected from the group consisting of heart, lungs, kidney, liver, and pancreas. Methods and compositions disclosed herein are applicable in humans. A suitable target site for example may require new vessel formation.

[0022] Endothelial colony forming cells include a density of about 1-2 million cells per milliliter volume of implantation support material. Other suitable ranges include 1-10 or 10-100 million cells per milliliter volume of implantation support material. Cultured ECFCs without any implantation material or matrix material may also directly administered or delivered to a target site. Concentration of ECFC may range from 1-10 or from 0.1-10 million cells/ml.

[0023] In an embodiment, the endothelial colony forming cells are cultured for about 12-48 hours prior to implantation. Other suitable incubation periods include for example, 6 hours, 10 hours, 15 hours, 20 hours, 36 hours, 42 hours, 48 hours, 3 days and 1 week. Suitable incubation temperatures include for example, 30-37° C., 24° C.

[0024] In an aspect, the support material is viscous and the support material provides three-dimensional cell growth. In an aspect, the support material that includes the cells is administered directly to the target site.

[0025] A method for forming functional blood vessels in vivo in a host, the method includes the steps of:

[0026] (a) culturing cord blood endothelial colony forming cells (ECFCs) in vitro;

[0027] (b) administering the cultured cells to a target site; and

[0028] (c) forming the vasculature in vivo in the host.

[0029] A method of augmenting blood supply at a target site, the method includes the steps of providing a matrix seeded with high proliferative-potential endothelial colony forming cells (HPP-ECFC) and inducing formation of a functional vasculature to increase blood supply at the target site.

[0030] A method of repairing an injury or preventing an injury to an endothelial surface, the method includes the steps of culturing high proliferative-potential endothelial colony forming cells (HPP-ECFC) and providing cultured endothelial colony forming cells at the endothelial surface to repair or prevent the injury to endothelial surface.

[0031] An implantable scaffold for treating an ischemic injury in a host, the scaffold includes high proliferative-potential endothelial colony forming cells (HPP-ECFC), wherein the endothelial colony forming cells associate to form blood vessels in vivo. The scaffold may also include a growth factor.

[0032] An engineered tissue includes a biologically compatible support material and a plurality of cultured high proliferative-potential endothelial colony forming cells (HPP-ECFC) that initiate vessel formation.

[0033] A tissue repair device includes an effective amount of high proliferative-potential endothelial colony forming cells (HPP-ECFC) culture on a biocompatible gel material. The tissue repair device includes one or more components of an extra cellular matrix. The tissue repair device includes endothelial colony forming cells that are cultured on a growth medium for endothelial cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0034] FIG. 1 shows a scheme for transplantation of cord blood ECFC. Cells ($1-2 \times 10^6$) derived from colonies of

circulating endothelial colony forming cells (ECFCs) established from human cord blood were implanted in 1 mL of type 1 collagen and fibronectin solution that hardens upon incubation at 37 degrees C. The gels are incubated in 24 well plates overnight. The ECFC containing gels are recovered from the plates and implanted subcutaneously in anaesthetized NOD/SCID mice. At 2-4 weeks later, the gels are imaged using a two photon microscope for visualization of blood flow in an anaesthetized animal. After the imaging the gels are recovered, fixed, and stained with monoclonal antibodies (anti-human CD31 or anti-mouse CD31) to identify the human vessels.

[0035] FIG. 2 shows (a) Human CD31 staining of collagen/FN gel implants; (b) is a higher magnification of (a). Antibody specific for human CD31 was used for the identification. Arrows indicate human cells expressing CD31. These cells are lining the surface of vessels and thus, are endothelial cells. The small round non-staining mouse red blood cells inside the vessels are shown.

[0036] FIG. 3 shows murine CD31 staining of collagen/FN gel implants. Arrows point to the mouse endothelial cells expressing CD31 in the subcutaneous tissues outside of the gels. No staining of vessels or endothelial cells inside of the gels indicates that the human cells are not stained by this antibody.

DETAILED DESCRIPTION

[0037] Human endothelial colony forming cells (ECFC), implanted subcutaneously in immunodeficient mice spontaneously form vessels that function to carry murine blood cells. A revised model for considering the cellular events involved in new vessel formation emphasizes the interacting role of macrophages and ECFC.

[0038] In an embodiment, methods of producing endothelial cell tubules or blood vessels in vivo include the steps of preparing a solution comprising collagen and fibronectin; suspending endothelial cells in the solution; warming the suspension so that the collagen gels to produce a three-dimensional gel; polymerizing the collagen within the solution to form a three-dimensional gel; and implanting the three-dimensional gel produced into an animal.

[0039] In an embodiment, methods and compositions are disclosed wherein the ECFCs associate to form structures characteristic of mature microvessels in vivo. The ECFCs form vasculature that are perfused by blood.

[0040] In an embodiment, methods and composition for promoting vascularization/revascularization in an animal include the steps of preparing a solution of a compatible biological polymer material and suspending ECFCs in the solution, wherein the suspended ECFCs associate in vitro; and directly injecting the solution into an animal of choice, including humans to form functional blood vessels in vivo.

[0041] Endothelial cell progenitor named a high proliferative potential-endothelial colony forming cell (HPP-ECFC) displays high proliferative potential (up to 100 population doublings compared to 20-30 doublings in adult blood EPC). HPP-ECFC were not only isolated from cord blood but from umbilical and adult blood vessels. HPP-ECFC cells can be replated at a single cell level and the majority of cells proliferate with regeneration of at least secondary HPP-ECFCs. Further, monolayers of cord blood endothelial cells

derived from HPP-ECFCs demonstrate a 2.5-fold decrease in population doubling times (PDT) and at least a 2-fold increase in cumulative population doubling levels (CPDL) compared to adult LPP-ECFCs (during the same time of culture ex vivo). In contrast to other populations of endothelial progenitor cells isolated from cord blood utilizing different methodologies, cord blood HPP-ECFC progeny uniformly express endothelial cell antigens and not hematopoietic specific cell antigens. Thus, HPP-ECFC are enriched in human umbilical cord blood and were not found in adult peripheral blood. HPP-ECFC were also found in endothelial cells derived from mammalian blood vessels e.g. umbilical vein and human aortic vessels. These HPP-ECFCs appear in cultures of freshly plated cord blood mononuclear cells within 10 days, whereas adult blood LPP-ECFCs rarely appear before 14 days after plating.

[0042] Cell cultures derived from HPP-ECFC are homogenous and display markers uniformly. These cells exhibit homogenous proliferative and clonogenic potential. Because some of these cell cultures are derived from a single HPP-ECFC, the resulting cells are homogenous and are not mixtures of cells.

[0043] Further, cord blood colonies consistently appeared larger compared to adult colonies. There were distinct differences in the size, frequency and time of appearance between adult and cord blood endothelial cell colonies. These observations show that cord blood EPCs are composed of HPP-ECFC, LLP-ECFC, and clusters, whereas adult blood EPCs are composed of LPP-ECFCs and clusters.

[0044] The complete hierarchy of HPP-ECFC, LPP-ECFC, clusters and mature endothelial cells can be isolated from any blood vessel in a living mammalian donor using the techniques described herein.

[0045] HPP-ECFCs give rise to all subsequent stages of endothelial progenitors in addition to replating into secondary HPP-ECFCs. Low proliferative potential-endothelial colony forming cells (LPP-ECFC) arising from single cells form colonies, which contain greater than 50 cells, but do not form at least secondary LPP-ECFC colonies upon replating. They do give rise to endothelial cell clusters (less than 50 cells). Finally, endothelial cell clusters can arise from a single cell but contain less than 50 cells, and do not replat into colonies or clusters.

[0046] Hematopoietic stem and progenitor cells are enriched in umbilical cord compared to adult peripheral blood. One intriguing observation was that EPCs were also enriched in umbilical cord blood compared to adult peripheral blood. Further, cord blood derived EPCs contain high levels of telomerase activity, which may account for the observation that these cells can be expanded for at least 100 population doublings without obvious signs of cell senescence. At the single cell level, some cord blood EPC-derived cells can be expanded 10^7 to 10^{12} fold.

[0047] Percent of dividing single cells giving rise to a colony with the number of cells in the quantitative ranges shown (HPP, LPP, clusters) (e) Representative photomicrographs (50x magnification) of the different endothelial cell clusters (<50 cells), LPP (about 51-2000 cells), and HPP (about 2000-to >10,000 cells) derived from a single cord blood or adult EPC-derived endothelial cell. Results are representative of 4 other independent experiments utilizing

cells from different donors. Scale bar in photomicrographs represents 100 μ m. LPP-ECFC (51-2000 cells) and HPP-ECFC (2000>10,000 cells) ranges are approximations.

[0048] The average level of telomerase activity in the adult samples was $4\pm 4\%$ and of the cord blood samples $34\pm 10\%$ of the telomerase activity of the HeLa cells. Comparison of telomerase activity of early and late passage adult and cord blood EPC-derived endothelial cells. PD indicates the cumulative population doubling level of the cells tested. P indicates telomerase activity in HeLa cells, which were used as a positive control. N indicates a negative control. Three other experiments utilizing early and late passage cord blood and adult EPC-derived endothelial cells from three different donors showed similar results.

[0049] The HPP-ECFC are small cells (nuclear diameter 8-10 microns) with minimal cytoplasmic spreading (diameters vary from 12-22 microns) with nuclear to cytoplasmic ratio >0.8 . LPP-ECFC are more heterogenous in size but are larger than HPP-ECFC. LPP-ECFC nuclei vary in size from 10.5-12.5 microns and have more cytoplasmic spreading (varying from 25-60 microns) with a ratio >0.4 but <0.5 . Endothelial clusters are nearly mature endothelial cells with nuclei that vary from 13.0-16.5 microns and have cytoplasmic diameters that vary from 65-80 microns and nuclear to cytoplasmic ratios of >0.2 but <0.3 . Mature differentiated endothelial cells are large very well spread cells with nuclear diameters that range from 17.0-22.0 microns and cytoplasmic diameters from 85-105 microns and nuclear to cytoplasmic ratios similar to endothelial clusters. Therefore, HPP-ECFC are very distinctly smaller than any of the other EPC and quite smaller than the mature endothelial cells

[0050] In an aspect, isolated endothelial colony forming cells have the following characteristics:

[0051] (a) express cell surface antigens that are characteristic of endothelial cells, such as CD31, CD105, CD146, and CD144;

[0052] (b) do not express cells surface antigens that are characteristic of hematopoietic cells, such as CD45 and CD14;

[0053] (c) ingest acetylated LDL; and

[0054] (d) form capillary- like tubes in Matrigel™ (extracellular matrix proteins).

[0055] The isolated cells classified as HPP-ECFC also

[0056] (a) replate into at least secondary colonies of at least 2000 cells when plated from a single cell;

[0057] (b) exhibit high proliferation;

[0058] (c) proliferate from a single cell; and

[0059] (d) express high levels of telomerase, at least 34% of that expressed by HeLa cells. HPP-ECFC also display a high nuclear to cytoplasmic ratio that is >0.8 , cell diameters <22 microns, and at least 10^7 progeny derive from a single cell.

[0060] A method of isolating endothelial colony cells includes steps of:

[0061] (a) culturing cells from a biological sample on supports coated with extracellular matrix proteins;

[0062] (b) selecting cells that adhere to the supports and form replatable colonies; and

[0063] (c) selecting single cells from the colonies.

[0064] The biological sample may be mammalian cord blood, or blood vessel. Human, bovine, porcine and rat sources are suitable. A single cell assay for types of endothelial cells includes the steps of:

[0065] (a) cell sorting of biological samples using a specific sorting method;

[0066] (b) culturing the single sorted cells on extracellular matrix protein under defined conditions; and

[0067] (c) enumerating specific colony sizes, morphology, and proliferative potential to determine the type of endothelial cell e.g. HPP-ECFC.

[0068] A method of enriching for HPP-ECFC includes the steps of:

[0069] (a) cell sorting of biological samples using a specific sorting method; and

[0070] (b) culturing of single sorted cells on extracellular matrix proteins under defined conditions.

[0071] A method for expanding hematopoietic stem cells ex vivo, include the steps of:

[0072] (a) culturing HPP-ECFC cells on collagen coated solid supports; and

[0073] (b) expanding hematopoietic stem cells (HSC) by co-culturing with HPP-ECFC cells wherein the HPP-ECFCs are derived from human cord blood cells and the HSC cells are derived from human bone marrow.

[0074] A method for improving the percentage of hematopoietic stem cells in a graft in a mammal, includes the step of:

[0075] (a) co-culturing human bone marrow cells with cord blood HPP-ECFC to form a product; and

[0076] (b) transplanting a suitable amount of the product into the mammal wherein the cells are CD45+ cells derived from human bone marrow, and the mammal is a NOD-SCID mouse.

[0077] Cord blood high proliferative potential—endothelial colony forming cells (HPP-ECFCs) in co-culture with autologous or unrelated cord blood, mobilized adult peripheral blood, or marrow-derived HSC expands the number of HSC cells and results in an increase in HSC and an increase in HSC repopulating activity leading to higher levels of engraftment in a recipient subject.

[0078] Use of a feeder layer of cells derived from high proliferative potential-endothelial colony forming cells (HPP-ECFCs) from human umbilical cord blood, stimulates growth and survival of repopulating hematopoietic stem and progenitor cells. Stimulation of growth and survival was determined by increased numbers of progenitor cells in in vitro culture and increased levels of human cell engraftment in the NOD/SCID immunodeficient mouse transplant system.

[0079] As used herein, the terms “encapsulated” and “embedded” refer to being entrapped and/or surrounded by a matrix or a support material. In the case of ECFCs,

encapsulated or embedded cells may be entirely or partially surrounded by matrix material; they may be able to move through, proliferate in and remodel the matrix.

[0080] “Matrix” as used herein, refers to the surrounding substance within which ECFCs associate or is contained. Artificial matrix refers to a matrix that is not naturally found.

[0081] “Three-dimensional cell culture” or “3-D cell culture” as used herein, refers to cell cultures wherein cell expansion can occur in any direction.

[0082] “Tissue cell culture” as used herein refers to an aggregation of cells and intercellular matter performing one or more functions in an organism. Examples of tissues include, but are not limited to, epithelium, connective tissues (e.g., bone, blood, cartilage), muscle tissue and nerve tissue.

[0083] “Two-dimensional cell culture” or “2-D cell culture” as used herein, refers to conventional monolayer cell culture. Generally, every cell in a 2-D culture directly contacts the substratum and the cultures, therefore, generally expand horizontally as they proliferate.

[0084] “Vascularization” as used herein, refers to the formation of new blood vessels. This includes formation of additional blood vessels from existing blood vessels. “Blood vessel” also includes capillary-like structures that are fully functional to support the transport of blood.

[0085] “Transplantation” as used herein, generally refers to the process by which a body part, organ, tissue or cell is transferred from one organism to another organism or transferred to an organism from an artificial source such as an organ or tissue harvested from cell or tissue culture systems. Grafts may include any tissue or body part or cells that are transferred to a desired site in vivo.

[0086] Support material as used herein refers to any biologically compatible substance that can support the association of ECFCs to form blood vessels. Suitable support material includes for example, biologically compatible polymer material selected from the group consisting of collagen, elastin, fibrinogen, fibrin, fibronectin, gelatin, laminin, vitronectin, hyaluronan, heparan sulfate, agar, agarose, alginate, chitosan and combinations thereof. Suitable support material can be selected from the group consisting of collagen-fibronectin, collagen-gelatin, collagen-agarose, collagen-chitosan, collagen-chitosan-agarose, collagen-chitosan-gelatin, collagen-vitronectin-agarose, collagen-vitronectin-gelatin, collagen-vitronectin-chitosan, collagen-fibronectin-agarose, collagen-fibronectin-gelatin, collagen-fibronectin-chitosan, collagen-laminin-agarose, collagen-laminin-gelatin, collagen-laminin-chitosan.

[0087] The support material or the implant material may further incorporate an additional agent selected from the group consisting of excipients, growth factors, vitamins, minerals, ions, gases, crosslinking agents, active agents, carriers and combinations thereof.

[0088] ECFCs are also implanted or ‘seeded’ into an artificial structure capable of supporting three-dimensional tissue formation. These structures, typically called scaffolds, are useful, both ex vivo as well as in vivo, to recapitulating the in vivo milieu and allowing cells to influence their own microenvironments. Scaffolds usually serve at least one of the following purposes: allow cell attachment and migration; deliver and retain cells and biochemical factors; enable

diffusion of vital cell nutrients and expressed products; exert certain mechanical and biological influences to modify the behavior of the cell phase. To achieve the goal of tissue reconstruction, scaffolds may meet some specific requirements. A high porosity and an adequate pore size are necessary to facilitate cell seeding and diffusion throughout the whole structure of both cells and nutrients. Biodegradability is a factor since scaffolds may be absorbed by the surrounding tissues without the necessity of a surgical removal. The rate at which degradation occurs may coincide with the rate of tissue formation: this means that while cells are fabricating their own natural matrix structure around themselves, the scaffold is able to provide structural integrity within the body and eventually it will break down leaving the neotissue, newly formed tissue which will take over the mechanical load. Injectability is also useful for clinical uses.

[0089] Many different materials (natural and synthetic, biodegradable and permanent) are useful. Biomaterials are engineered to have suitable properties and functional customization: injectability, synthetic manufacture, biocompatibility, non-immunogenicity, transparency, nano-scale fibers (nanomaterial), low concentration, resorption rates.

[0090] Scaffolds or implantable material may also be constructed from natural materials:

[0091] in particular different derivatives of the extracellular matrix are suitable for their ability to support cell growth. Proteic materials, such as collagen or fibrin, and polysaccharidic materials, like chitosan or glycosaminoglycans (GAGs) are suitable.

[0092] As used herein, “consisting essentially of” is intended to mean that the composition includes components that are primarily responsible for blood vessel formation, e.g., ECFCs and any other component that does not materially affect the formation of blood vessels in vivo.

[0093] The content and teaching of co-pending applications U.S. Ser. No. 60/822,166 filed Aug. 11, 2006, U.S. Ser. No. 11/055,182 filed Feb. 9, 2005, U.S. Ser. No. 60/543,114 filed Feb. 9, 2004, U.S. Ser. No. 60/542,949 filed Feb. 9, 2004, U.S. Ser. No. 60/573,052 filed May 21, 2004 and U.S. Ser. No. 60/637,095 filed Dec. 17, 2004 are hereby expressly incorporated by reference as they relate to the isolation and characterization of endothelial colony forming cells. Vessel formation in fibronectin gel is for example, described in U.S. Publication No. 20040072342, the disclosure of which is hereby incorporated by reference. Some examples of engineering tissues can be found in U.S. Publication Nos. 20050031598, and 20040009589, the disclosures of which are incorporated by reference.

EXAMPLES

[0094] The following examples are illustrative embodiments and are not intended to limit the scope of the disclosure.

Example 1

Characterization of Endothelial Cell Colonies (EPCs) Isolated from Human Umbilical Cord Blood

[0095] Cells were expanded in culture while maintaining an endothelial cell phenotype.

[0096] In contrast to previously described endothelial progenitor cells isolated from cord blood, the present disclosure relates that cells isolated from human umbilical cord blood, i.e. cord blood HPP-ECFCs and progeny, can be cultured for at least 100 population doublings and expanded exponentially even when beginning with a single cell. Further, these cells do not express the hematopoietic cell specific surface antigens, CD45 and most also do not express CD14, and do not form hematopoietic cell colonies in methycellulose assays. In addition, the HPP-ECFC progeny rapidly form vessels in Matrigel™ (extracellular matrix proteins), upregulate VCAM-1 in response to either IL-1 or TNF- α stimulation, and express endothelial cell specific antigens, which confirms their endothelial cell identity. These cells were designated high proliferative potential-endothelial colony forming cells (HPP-ECFCs).

[0097] Cord blood HPP-ECFCs demonstrate greater replicative kinetics compared to adult blood (which are composed of LPP-ECFC, clusters, and mature endothelial cells). While endothelial progenitors are reported to express AC133, CD34, and Flk1, HPP-ECFC progeny and EOC progeny display similar frequencies of cells expressing AC133, CD34, and Flk1 antigens and, therefore, these cell surface markers do not permit discrimination of cells with differing proliferative potentials.

[0098] Endothelial outgrowth cells appear two to four weeks after culture of MNCs isolated from adult peripheral blood and are characterized by their exponential growth in vitro (however, EOC display lower levels of telomerase, do not replete into secondary LPP-ECFC, and reach replicative senescence long before TPP-ECFC). In contrast, HPP-ECFC generated from human umbilical cord blood MNCs, emerged five to ten days after culture of cord blood MNCs in complete EGM-2 media on tissue culture plates coated with type I collagen. Discrete adherent cell colonies appeared and displayed the “cobblestone” morphology of endothelial cells. The morphology and appearance of the colonies was similar to, but distinct from, that previously described for adult peripheral blood derived EOC colonies and clearly distinct from adherent circulating endothelial cells or macrophages. The HPP-ECFC colonies are large and are composed of a mixture of small round, long thin, and large flattened round cells whereas the EOC are nearly homogeneously composed of long thin cells. The colony-derived cells were subcultured and expanded cells derived from these colonies were used for immunophenotyping, functional testing and measurement of growth kinetics. After initial passage, the cells formed monolayers of spindle shaped cells with “cobblestone” morphology. Immunophenotyping revealed that the cells uniformly expressed the endothelial cell surface antigens, CD31, CD141, CD105, CD146, CD144, vWF, and flk-1. The cells did not express the hematopoietic cell surface specific antigens, CD45 and CD14, confirming that the monolayers were not contaminated with hematopoietic cells.

[0099] Confirming that the monolayers derived from the adherent colonies were endothelial cells, the cells ingested acetylated-low density lipoprotein (Ac-LDL) or (Dil-Ac-LDL). These cellular functions are characteristic of endothelial cells. Cells subcultured from the adherent colonies uniformly incorporated Ac-LDL, formed vessels in Matrigel™ (extracellular matrix proteins) after seeding varying

numbers of cells, and upregulated VCAM-1 in response to both rhTNF- α or rhIL-1 stimulation.

[0100] The growth kinetics of cord blood HPP-ECFC and progeny were measured as a function of time. Strikingly, cord blood HPP-ECFC and progeny could be exponentially expanded in culture for at least 100 population doublings without signs of senescence, and the number of cells increased 10^{20} fold over a period of 100 days in culture. A representative growth curve of cord blood endothelial cells, illustrates the proliferative potential of these cells in vitro. Thus, based on immunophenotyping, functional testing, and an analysis of growth kinetics, it was shown that colonies of endothelial cells (designated HPP-ECFCs) can be uniquely cultured from cord blood MNCs and passaged into confluent monolayers of exponentially expandable endothelial cells.

Example 2

HPP-ECFC Colonies are Present in Human Umbilical Cord Blood but Not in Adult Peripheral Blood

[0101] 50-100 milliliters of peripheral blood was collected from healthy adult donors or from umbilical cords of normal term infants and isolated MNCs. Cells were seeded into tissue culture plates coated with extracellular mature molecules in complete EGM-2 media and observed for colony formation over the next one to six weeks. The number of colonies per equivalent volume of blood was increased 15 fold in cord blood compared to adult peripheral blood (Table I). Similar differences in colony formation were also observed when equivalent numbers of cord and adult MNCs were plated. Although adult EOC colonies typically formed between 2-4 weeks after initiation of culture, cord blood HPP-ECFC colonies appeared within 5-10 days. Finally, immunophenotyping of the cells isolated from endothelial colonies from both cord blood and adult peripheral blood by flow cytometry revealed that the colony-cells uniformly expressed the endothelial cell surface antigens, CD31, CD105, CD146, CD144, vWF, and flk-1 and not the hematopoietic cell surface antigens CD45 and CD14, confirming their endothelial cell identity. Immunophenotyping of the adult EOCs was consistent with previously published studies. Thus, endothelial colony forming cell HPP-ECFC are present in cord blood and represent a different cell type compared to adult peripheral blood EOCs (which represent LPP-ECFCs).

Example 3

The Proliferative Rate of Cord Blood HPP-ECFCs is Greater than Adult Blood EOCs

[0102] Given the differences in the frequency and the time of appearance of colony formation of cells from cord blood compared to adult peripheral blood, a question was whether there were differences in the proliferative kinetics of adult and cord blood cells. Early passage monolayers of HPP-ECFC were established from cord blood and EOC established from adult peripheral blood, and cells were cultured in complete EGM-2 media on type I collagen coated plates. Input cell numbers were counted for determination of population doubling times (PDT) and cumulative population doubling levels (CPDL) in long-term cultures, which were measurements used to quantitate and to compare the proliferative

erative kinetics of cord blood and adult blood derived cells. Cells were cultured for at least 10 passages to accurately quantitate the PDT and CPDL. Results testing multiple cell lines from different donors, showed that there was a 2.5 fold decrease in the PDT of cord blood HPP-ECFCs compared to adult EOC controls. Further, consistent with a decrease in PDT, culture of cord blood cells demonstrated a significant increase in CPDLs compared to serial passage of adult EOCs. Thus, although both cord and adult cells can be expanded in culture, the proliferative potential of cord blood HPP-ECFC and progeny is greater compared to adult EOC. Cord blood derived HPP-ECFC also demonstrate greater proliferative potential at the single cell level compared to adult blood EOCs.

[0103] The proliferative and clonogenic capacity of individual cord blood HPP-ECFC-derived endothelial cells or adult EOCs at the single cell level was determined. A novel experimental method was designed to quantitate the proliferative and clonogenic capacity of single cord blood HPP-ECFC-derived endothelial cells and adult EOCs.

[0104] Early passage cord blood HPP-ECFC-derived endothelial cells or adult EOC progeny were initially transduced with a retrovirus encoding a green fluorescent protein (GFP) and selected for expression of GFP. Transduction efficiency of both cord and adult endothelial cells was greater than 95%. Following selection, one GFP expressing HPP-ECFC-derived endothelial cell or adult EOC-derived endothelial cell was plated by fluorescent cytometry sorting (using a sorting nozzle with a diameter ≥ 100 microns and a sheath flow pressure of ≤ 9 pounds per square inch) into one well of a 96 well tissue culture plate coated with type I collagen and filled with 200 μ l of EGM-2 media. Immediately following placement, individual wells were examined to ensure that only one endothelial cell had been placed into each well. Endothelial cells were then cultured for 14 days, and one half of the media was changed every 4 days with fresh EGM-2 media. At the end of 14 days, the number of GFP expressing endothelial cells was counted.

[0105] The number of single cells undergoing at least one cell division was significantly greater for cord blood HPP-ECFC-derived endothelial cells compared to adult EOC-derived endothelial cells. In scoring the number of cells in each well at the end of 14 days, it was clear that single cord blood HPP-ECFC-derived endothelial cells divided more and produced larger colonies compared to adult EOC-derived endothelial cells. Because of differences in the capacity of single cord blood HPP-ECFC-derived endothelial cells to divide and form colonies compared to adult EOC-derived endothelial cells, the number of cells in each well, which demonstrated at least one cell division, were counted. Although, most of the single adult EOC-derived endothelial cells (which had divided), produced clusters of between 2 and 50 cells, some did give rise to secondary colonies of up to 500 cells, but only a single colony of >2000 cells arose from any of the single sorted adult EOC-derived endothelial cells. However, greater than 60% of the cord blood HPP-ECFC-derived endothelial cells (which had divided), formed well circumscribed secondary colonies consisting of at least 2000 cells, and numerous single sorted cells gave rise to colonies composed of >10,000 cells (to the inventors' knowledge, no adult EOC-derived endothelial cells ever produced such a colony).

[0106] Secondary cell colonies derived from either single adult EOC-derived or cord blood HPP-ECFC-derived endothelial cells were serially replated to determine if these cells could form more colonies. Secondary colonies derived from single adult EOC-derived endothelial cells never gave rise to tertiary colonies after replating in 24 well or 6 well type I collagen coated tissue culture plates in multiple independent experiments. Single cells plated remained quiescent and did not proliferate. However, most of the secondary colonies derived from single cord blood HPP-ECFC-derived endothelial cells, which produced greater than 2000 cells, could be replated under the same experimental conditions to form tertiary endothelial colonies. Single primary cord blood HPP-ECFC-derived endothelial cells can produce secondary colonies, which can be subsequently serially passaged to produce from 10^7 - 10^{12} endothelial cells.

[0107] Given the similarities of this unique and newly identified population of cord blood derived endothelial colony forming cells to the hematopoietic high proliferative potential-colony forming cells (HPP-CFC; the most primitive multipotent hematopoietic progenitor that can be cultured in an in vitro clonogenic assay) these cells are named "high proliferative potential-endothelial colony forming cells (HPP-ECFC)". In summary, these cells are different from the previously described adult EOCs in the following ways: (1) HPP-ECFCs have higher proliferative kinetics when cultured under the same experimental conditions as adult EOCs, (2) HPP-ECFCs appear at earlier timepoints in culture from plated cord blood MNCs compared to adult EOCs derived from plated adult peripheral MNCs, (3), HPP-ECFCs have higher clonogenic potential at the single cell level compared to adult EOCs. (4) HPP-ECFCs can be serially replated to form at least secondary HPP-ECFC colonies while/whereas adult EOCs do not display this potential, and HPP-ECFC display high levels of telomerase.

Example 4

Growth Kinetics of EPC-Derived Cord Blood and Adult Endothelial Cells

[0108] Progenitor cells of different lineages are defined and discriminated by their clonogenic and proliferative potential. Because of the differences in cord blood and adult EPC colony formation, the proliferative kinetics of EPC-derived cord blood and adult endothelial cells were compared. Initially cells derived from cord blood and adult endothelial cell colonies were plated at limiting cell dilutions to test whether the cells would form secondary colonies and grow to confluence. Interestingly, the cell progeny derived from both adult and cord blood EPC colonies formed secondary cell colonies of various sizes before growing to confluence. However, colonies derived from cord blood EPC-derived cell progeny were consistently larger and contained smaller cells compared to adult colonies.

[0109] Cell monolayers were serially passaged to determine the proliferative potential of EPC-derived cord blood and adult endothelial cells. Remarkably, cord blood EPC-derived cells could be expanded for at least 100 population doublings without obvious signs of senescence. In contrast, adult EPC-derived cells could be passaged for only 20-30 population doublings. To quantitate and compare the proliferative kinetics of cord blood and adult EPC-derived cells,

the population doubling times (PDT) and cumulative population doubling levels (CPDL) were calculated during a defined time in culture (60 days). There was a 2.5 fold decrease in the PDT and a 1.5 fold increase in the CPDLs of cord blood EPC-derived cells compared to adult EPC-derived cells. The PDT and CPDL of adult EPCs was similar to two recent reports, which tested the proliferative kinetics of EPC-derived cells isolated from healthy adult donors.

[0110] The proliferation of cord blood and adult EPC-derived cells in response to either rhVEGF or rhbFGF stimulation, which are two endothelial cell mitogens were compared. Cord blood and adult EPC-derived cells were serum starved and then cultured in the presence or absence of either rhVEGF or rhbFGF. Cells were cultured for 16 hours, and pulsed with tritiated thymidine before harvest to measure DNA synthesis. Cord blood EPC-derived cells displayed greater DNA synthesis in response to either rhVEGF or rhbFGF stimulation compared to adult EPC-derived cells. Collectively, these results demonstrate that the proliferative rate and expandability of cord blood EPC-derived cells is greater than adult EPC-derived cells in both short and long term assays. Further, cord blood and adult EPC-derived endothelial cells form distinct cell colonies of various sizes and morphology when plated at limiting dilution.

Example 5

Quantitation of the Clonogenic and Proliferative Potential of Single Cord Blood and Adult Endothelial Cells Derived from EPC Colonies

[0111] Cord blood and adult EPC colonies yield cells with different proliferative and clonogenic potential. However, a rigorous test for the clonogenic potential of a progenitor cell is to determine whether a single cell will divide and form a colony in the absence of other cells. Therefore, an assay was developed to quantitate the proliferative and clonogenic potential of single cord blood and adult endothelial cells derived from EPC colonies.

[0112] Cord blood and adult endothelial cells derived from the initial EPC colonies were transduced with a retrovirus encoding EGFP and selected for EGFP expression. Following selection, one EGFP expressing endothelial cell was plated by FACS into one well of a 96 well tissue culture plate coated with type I collagen and filled with complete EGM-2 media. Endothelial cells were cultured, and the number of EGFP-expressing endothelial cells was counted at the end of 14 days as disclosed herein.

[0113] The percentage of single cells undergoing at least one cell division was increased five fold for cord blood endothelial cells compared to adult cells. Further, the average number of cell progeny derived from a single cord blood endothelial cell was 100 fold greater compared to the number of cells derived from an individual adult cell. Greater than 80% of the single adult endothelial cells which divided gave rise to small colonies or clusters of cells ranging in number from 2-50 cells. However, some single adult endothelial cells did form colonies containing greater than 500 cells. In contrast, at least 60% of the single plated cord blood endothelial cells which divided formed well-circumscribed colonies containing between 2,000 and 10,000 cells in the 14 day culture period. The single cell

studies demonstrate that there are different types of cord and adult EPCs, which can be discriminated by their proliferative and clonogenic potential, and that EPCs display a hierarchy of proliferative potentials similar to the hematopoietic progenitor cell hierarchy.

Example 6

The Cell Progeny of Single Cord Blood Endothelial Cells can be Serially Replated and Expanded Exponentially in Long-Term Cultures

[0114] In the hematopoietic cell system, the most proliferative progenitor cell type is termed the high proliferative potential-colony forming cell (HPP-ECFC). The HPP-ECFC is defined by its ability to form large cell colonies, which yield individual cells that have the potential to form at least secondary colonies upon serial replating. The clonal progeny derived from a single plated cord blood or adult EPC-derived cell were trypsinized, replated and cultured into 24-well tissue culture plates for 7 days. After plating the clonal progeny of over 1000 single adult EPC-derived cells into 24 well plates, only one secondary colony was detected in the wells after 14 days of culture. In contrast, approximately one half (205 of 421) of the clonal progeny of single plated cord blood EPC-derived cells formed secondary colonies or rapidly grew to confluence in 24 well plates. Since secondary colonies were not detected in those wells that had rapidly grown to confluence in 5 days, a limiting dilution analysis was performed on the confluent monolayer. At least nine percent of the single cells plated from this monolayer formed an endothelial cell colony, containing greater than 100 cells. This result verifies that individual cells derived from cord blood EPCs are capable of forming secondary colonies.

[0115] The long-term proliferative potential of the cells derived from a single plated cord blood EPC-derived endothelial cell was tested. Secondary colonies or confluent cell monolayers derived from single cord blood endothelial cells were serially passaged into progressively larger tissue culture plates. The cell progeny of 11 single endothelial cells, originally derived from three different cord blood donors were tested. Single cord blood endothelial cells yielded at least 10^7 cells in long-term culture. The average CPDL of the eleven single cord blood endothelial cells tested was 30.8. Thus, a population of high proliferative EPCs in cord blood, which form secondary and tertiary colonies.

Example 7

EPC-Derived Cord Blood Endothelial Cells Contain High Levels of Telomerase Activity

[0116] Endothelial cells derived from cord blood EPCs were serially passaged beyond Hayflick's limit for at least 100 population doublings. The only other reported primary endothelial cells with similar growth kinetics are those genetically engineered to overexpress telomerase. Thus, telomerase activity was measured in cord blood and adult EPC-derived cells as a potential molecular explanation for the differences in their growth kinetics. Both early and late passage cord blood EPC-derived progeny display significantly elevated levels of telomerase activity compared to adult EPC-derived cells, reminiscent of the previously described primary endothelial cells lines, which overexpress

telomerase. Thus, consistent with extensive proliferative potential, cord blood EPC-derived cells retain high levels of telomerase activity ($34 \pm 10\%$ of the telomerase activity of an equal number of HeLa cells) with serial passage in culture.

Example 8

Expansion of Repopulating Stem and Progenitor Cells Ex Vivo

[0117] Cord blood high proliferative potential—endothelial colony forming cell (HPP-ECFC) in co-culture with autologous or unrelated cord blood, mobilized adult peripheral blood, or marrow-derived HSC, expands the number of HSC cells and results in an increase in HSC and an increase HSC repopulating activity leading to higher levels of engraftment in a recipient subject.

[0118] Co-culture of HPP-ECFC from cord blood with human HSC increases hematopoietic progenitor cell numbers and enhances engraftment of human hematopoietic cells in NOD/SCID mice, an assay for in vivo measure of human HSC function.

[0119] Human cord blood HPP-ECFC-derived endothelial cells co-cultured with human cord blood or mobilized adult peripheral blood CD34⁺CD38⁻ cells (enriched in HSC activity) for up to 7 days (with added cytokines) results in an enhancement in human CD45⁺ cell engraftment in sublethally irradiated NOD/SCID mice by >100 fold.

[0120] A method of collection, isolation, and expansion of the HPP-ECFC and the particular method for co-culturing the HPP-ECFC with human stem cells are novel. HPP-ECFC can be collected from any cord blood sample, expanded, frozen, and stored. These cells can then be thawed, expanded, and used in co-culture to expand human cord blood, marrow-derived, or mobilized adult peripheral blood stem and progenitor cell samples. The expanded product can then be used for transplantation purposes (after regulatory agency approval).

Example 9

Vessel Formation Using ECFCs

[0121] This example demonstrates the vessel forming capability of human cord blood derived endothelial colony forming cells (ECFCs) that are established in cell culture. A general scheme is illustrated in FIG. 1 and the identification of vessel formation in FIGS. 2-3. The ECFCs are isolated using the methods disclosed herein and are then suspended in collagen and/or fibronectin gels for vessel formation. In one aspect, 10^6 ECFCs/ml of gel material are used. The ECFCs in the gels are cultured for about 12-24 hours in an incubator. The ECFCs in the gels initiate vessel formation by initiating association among the cells. The gel implants with cultured ECFCs are then implanted at a desired site. Vessel formation is analyzed by blood flow analysis (e.g., confocal imaging or immuno histochemistry, IHC), if necessary. Further staining by CD31 markers are also performed to verify the presence of blood vessel formation. FIGS. 2-3 show human CD31 staining of collagen/FN gel implants. The vessel formation is indicated by arrows. The amount of cells and/or the nature of the gel material used may vary depending on the treatment site and the nature of the

therapeutic process. For example, ECFCs in the range of about 10^5 - 10^7 /ml of gel, or 10^4 - 10^8 /ml of gel are suitable for vessel formation in vivo.

[0122] In an embodiment, the ECFCs are cultured and are directly introduced to a target site or in the circulatory system without the presence of any support material, e.g., matrix material. The ECFCs may adhere to the target site and associate to form blood vessels or otherwise repair the endothelial lining. In an embodiment, the associated ECFCs are introduced to a target site by direct administration of cultured ECFCs.

Example 10

Clinical Therapy Using Cultured ECFCs to Enhance Blood Vessel Formation

[0123] This example demonstrates the use of cultured ECFCs for a variety of clinical applications. Clinical indications for ECFC therapy to enhance blood vessel formation to alleviate ischemia and hypoxia to tissues would include individuals with peripheral arterial disease. This disorder strikes middle-aged and elderly individuals and patients that smoke or suffer from diabetes are more susceptible. Diminished arterial blood flow, particularly in the lower extremities, can lead to pain with walking and eventual inability to walk, tissue necrosis, and need for amputation.

[0124] Use of ECFC via direct administration into an ischemic site would form new vessels in the affected areas, improve blood flow, and salvage the extremities. In diabetic patients with poor wound healing and diminished blood flow, ECFC administration into the sites enhance the overall blood flow and improve the healing process. Patients requiring skin and/or bone tissue grafts following major trauma or repair of tissue defects following cancer and chemotherapy benefit by improved graft perfusion and faster tissue repair. Another population likely to benefit from ECFC therapy include patients with myocardial ischemia. Administration of cultured ECFCs, for example, in a gel, into the ischemic region enhances recovery of perfusion and salvage cardiomyocytes with overall improved long term outcomes.

[0125] Any clinically significant condition that can be alleviated by increased blood supply through formation new blood vessels or through repairing of existing blood vessels are treated with the ECFCs described herein.

Materials and Methods

Adult Peripheral and Umbilical Cord Blood Samples

[0126] Fresh blood samples (50-100 ml) were collected by venipuncture and anticoagulated in citrate phosphate dextrose solution from healthy human volunteers (males and females between the ages of 22 and 50). Human umbilical cord blood samples (20-70 ml) from healthy newborns (38-40 weeks gestational age, males and females) were collected in sterile syringes containing citrate phosphate dextrose solution as the anticoagulant. Written informed consent was obtained from all mothers before labor and delivery. The Institutional Review Board at the Indiana University School of Medicine approved all protocols.

Buffy Coat Cell Preparation

[0127] Human mononuclear cells (MNCs) were obtained from either adult peripheral or umbilical cord blood. Briefly,

20-100 ml of fresh blood was diluted one to one with Hanks Balanced Salt Solution (HBSS) (Invitrogen, Grand Island, N.Y.) and overlayed onto an equivalent volume of Ficoll-Paque (Amersham Biosciences) a ficoll density gradient material. Cells were centrifuged for 30 minutes at room temperature at 1800 rpms (740×g). MNCs were isolated and washed three times with EBM-2 medium (Cambrex, Walkersville, Md.) supplemented with 10-20% fetal bovine serum (Hyclone, Logan, Utah), 2% penicillin/streptomycin (Invitrogen) and 0.25 µg/ml of amphotericin B (Invitrogen) (complete EGM-2 medium).

Culture and Quantitative Analysis of Endothelial Outgrowth Cells

[0128] Buffy coat MNCs were initially re-suspended in 12 ml of EGM-2 medium (Cambrex) supplemented with 10% fetal bovine serum, 2% penicillin/streptomycin and 0.25 µg/ml of amphotericin B (complete EGM-2 medium). Four milliliters of cells were then seeded onto three separate wells of a six well tissue culture plate (BD Biosciences, Bedford Mass.) previously coated with extra cellular matrix proteins e.g. type I rat tail collagen (BD Biosciences) vitronectin, fibronectin, collagen type 10, polylysine. The plate was incubated at 37° C., 5% CO₂ in a humidified incubator. After 24 hours of culture, the non-adherent cells and debris were carefully aspirated, and the remaining adherent cells were washed one time with 2 ml of EGM-2 medium. After washing, 4 ml of EGM-2 medium was added to each well. EGM-2 medium was changed daily until day 7 of culture and then every other day until the first passage.

[0129] Colonies of cells initially appeared between 5 days and 22 days of culture and were identified as well circumscribed monolayers of cobblestone appearing cells. Colonies were enumerated by visual inspection using an inverted microscope at 40× magnification.

[0130] For passaging, cells were removed from the original collagen coated tissue culture plates using 0.05% trypsin-0.53 mM EDTA (Invitrogen), resuspended in 10 ml of EGM-2 media and plated onto 75 cm² tissue culture flasks coated with type I rat tail collagen. Monolayers of endothelial cells were subsequently passaged after becoming 90-100% confluent.

Culture of HUVECs and HAECs

[0131] Two approaches were used to directly isolate the endothelial cells from arterial or venous vessels. In the first approach, a 20 G blunt end needle was inserted into one end of an incised vessel and the vascular contents (plasma with blood cells) were flushed out the opposite end using sterile saline. Vascular clamps were then applied to isolate each end of the vessel (3-5 cm in length). A solution of 0.1% collagenase in Hanks balanced salt solution (HBSS) was injected through the vessel wall via a 23 G needle, and the vessel segments were incubated for 5 min at 37° C. The vascular clamp from one end of the vessel was then removed and the endothelial cells were expelled via infusion of a cell dissociation buffer (Gibco) (injected through the distal end of the vessel opposite the "open" end of the vessel). The vessel segments were infused with a minimum of 10 mL of cell dissociation buffer. The suspended cells were centrifuged at 350×g and washed in EBM-2 media with 10% FBS, counted, and viability checked using Trypan blue exclusion.

[0132] The second approach is best suited for large diameter vessels (>1 cm). The vessel was incised along the entire

length and opened with the endothelial lumen exposed. Any remaining blood cells and plasma were washed away with HBSS. The endothelium was removed by firm scraping with a rubber policeman in a single end-to-end motion. The cells adhering to the rubber policemen were washed free by swirling the policemen in a solution of EBM-2 with 10% FBS in a 6 cm tissue culture well (precoated with extracellular matrix proteins). Cells were cultured with visual examination each day. Colonies of endothelium emerge in 3-10 days. The adherent endothelial colonies were removed by trypsin-EDTA and transferred to T 25 flasks that were coated with extracellular matrix proteins. Cryopreserved human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs) were obtained from Cambrex at passage three. Cells were seeded in 75 cm² tissue culture flasks precoated with type I rat tail collagen in complete EGM-2 medium for passage.

Growth Kinetics and Estimate of Replicative Capacity of EPCs.

[0133] At the time of first passage cells were enumerated by a trypan blue exclusion assay (Sigma, St. Louis, Mo.). Monolayers of cells were then grown to 90% confluence and passaged. At each passage, cells were enumerated for calculation of a growth kinetic curve, population doubling times (PDTs), and cumulative population doubling levels (CPDLs).

[0134] The number of population doublings (PDs) occurring between passages was calculated according to the equation: $PD = \log_2 (C_H/C_S)$ where C_H is the number of viable cells at harvest and C_S is the number of cells seeded. The sum of all previous PDs determined the CPDL at each passage. The PDT was derived using the time interval between cell seeding and harvest divided by the number of PDs for that passage. Matrigel™ (extracellular matrix proteins) assays and uptake of acetylated-low density lipoprotein (Ac-LDL or Dil-Ac-LDL)

[0135] Matrigel™ (extracellular matrix proteins) assays were performed. Briefly, early passage (2-3) HPP-ECFC-derived or EPC-derived endothelial cells were seeded onto 96 well tissue culture plates previously coated with 30 µl of Matrigel™ (extracellular matrix proteins) (BD Biosciences) at a cell density of 5000-20,000 cells per well. Cells were observed every two hours for capillary-like tube formation.

[0136] To assess the ability of attached HPP-ECFC and progeny or EPC and progeny to incorporate Ac-LDL or Dil-Ac-LDL, 10 µg/ml of Ac-LDL (Biomedical Technologies Inc., Stoughton, Mass.) was added to the media of cells cultured in a 6 well type I rat tail collagen coated tissue culture plate. Cells were incubated for 30 minutes or 4 hours at 37° C. and then washed three times with phosphate buffered saline (PBS) stained with 1.5 µg/ml of DAPI (Sigma) and examined for uptake of Ac-LDL or Dil-Ac-LDL by using a fluorescent microscope.

Immunophenotyping of Endothelial Cells by Fluorescence Cytometry

[0137] Early passage (1-2) or (3-4) HPP-ECFC and progeny or EPC and progeny (5×10^5) were incubated at 4° C. for 30-60 minutes with varying concentrations of the primary or isotype control antibody as outlined below in 100 µl of PBS and 2% FBS. Cells were washed three times with PBS containing 2% FBS and analyzed by fluorescence activated

cell sorting (FACS®) (Becton Dickinson, San Diego, Calif.). Directly conjugated primary murine monoclonal antibodies against human CD31 conjugated to fluorescein isothiocyanate (FITC) (BD Pharmingen, San Diego, Calif.) were used at a 1:20 dilution, human CD34 conjugated to allophycocyanin (APC) (BD Pharmingen) at a 1:25 dilution, human CD14 conjugated to FITC (BD Pharmingen) at a 1:10 dilution, human CD45 conjugated to FITC (BD Pharmingen) at a 1:10 dilution, human CD117 conjugated to APC (BD Pharmingen) at a 1:100 dilution, human CD146 conjugated to phycoerythrin (PE) (BD Pharmingen) at a 1:10 dilution, human AC133 conjugated to PE (Miltenyi Biotec, Auburn, Calif.) at a 1:5 dilution, human CD141 conjugated to FITC (Cymbus Biotechnology, Chandlers Ford, UK) at a 1:10 dilution, human CD105 (BD Pharmingen) conjugated to Alexa Fluor 647 (Alexa Fluor 647 monoclonal antibody labeling kit, Molecular Probes, Eugene, Oreg.) at a 1:100 dilution, and human CD144 conjugated to Alexa Fluor 647 at a 1:100 dilution.

[0138] To test for cell surface expression of vascular cell adhesion molecule (VCAM-1) after activation by a cell agonist, serum starved endothelial cells were stimulated with either 10 ng/ml of recombinant human interleukin one (IL-1) (Peprotech, Rocky Hill, N.J.) or 10 ng/ml of recombinant human tumor necrosis factor- α (TNF- α) (Peprotech) for 4 hours at 37° C. Following stimulation, cell surface expression of VCAM-1 was tested utilizing a primary antibody against human VCAM-1 conjugated to FITC (BD Pharmingen) at a 1:20 dilution. For all isotype controls for immunophenotyping and VCAM-1 expression, the following antibodies were used: mouse IgG_{2a}, κ , conjugated to FITC (BD Pharmingen), mouse IgG₁, κ conjugated to FITC (BD Pharmingen), mouse IgG₁, κ conjugated to PE (BD Pharmingen), and mouse IgG₁, κ conjugated to APC (BD Pharmingen).

[0139] For detection of cell surface expression of von Willebrand factor (vWF) and flk-1, cells were fixed in acetone for 10 minutes at room temperature, washed two times with PBS, and blocked and permeabilized for 30 minutes with PBS, 3% nonfat dry milk, and 0.1% Triton X-100 (Sigma). We used 2 μ g/ml of a primary antibody directed against human vWF (Dako, Carpinteria, Calif.) and a biotinylated primary antibody directed against human flk-1 (Sigma) at a 1:20 dilution. The secondary antibody used for vWF was a goat anti-rabbit antibody conjugated to FITC (BD Pharmingen) at a 1:100 dilution and the secondary antibody used for flk-1 was streptavidin conjugated to APC (BD Pharmingen) at a 1:100 dilution. For the isotype control for vWF, we used rabbit Ig primary antibody (Dako) at a 1:100 dilution with anti-rabbit Ig secondary antibody conjugated to FITC (BD Pharmingen) at a 1:100 dilution. For the isotype control for flk-1, we used a biotinylated mouse IgG₁, κ (BD Pharmingen) primary antibody at a 1:100 dilution with a streptavidin APC secondary antibody (BD Pharmingen) at a 1:100 dilution.

Telomerase Activity Assay

[0140] For detection of telomerase activity, the telomeric repeat amplification protocol (TRAP) was employed in the form of a TRAP-eze telomerase detection kit (Oncor, Gaithersburg, Md.). Briefly, 1000 cultured HPP-ECFC or EPC colonies were absorbed onto filter papers and lysed in TRAP assay buffer. The lysed material was subjected to PCR

amplification and the PCR products (6-bp incremental ladder) were electrophoresed on a non-denaturing polyacrylamide gel and visualized by DNA staining or radiolabeled with ³²P. PCR products were loaded as neat or 1/10 or 1/100 dilutions and the level of intensity of staining compared to the HELA cell line (1000 cells) positive control.

Thymidine Incorporation Assays

[0141] Endothelial colony-derived endothelial cells were deprived of growth factors and cultured in EBM-2 media supplemented with 5% FBS for 24 hours. Next, 3 $\times$ 10⁴ cells were plated in each well of 6-well tissue culture dishes pre-coated with type I collagen and cultured for 16 hours in EBM-2 media supplemented with 1% FBS. Cells were then cultured in EBM-2 without serum for an additional eight hours to ensure quiescence. Cells were stimulated in EBM-2 media supplemented with 10% FBS with 25 ng/ml of recombinant human vascular endothelial growth factor (rhVEGF) (Peprotech), 25 ng/ml of recombinant human basic fibroblast growth factor (rhbFGF) (Peprotech) or no growth factors, as indicated, in a 37° C., 5% CO₂, humidified incubator. Some cells were cultured in EBM-2 media without growth factors or FBS. Cells were cultured for 16 hours, and 1 μ Ci of tritiated thymidine (Perkin Elmer Life Sciences Products, Boston, Mass.) was added 5 hours prior to the harvest. Cells were lysed with 0.1 N sodium hydroxide for one hour. Lysates were collected into 5 ml of liquid scintillant (Fisher Scientific, St. Louis, Mo.) and β emission was measured. Assays were performed in triplicate.

Generation of GALV-Pseudotyped MFG-EGFP

[0142] The MFG-EGFP retrovirus vector expresses the enhanced green fluorescent protein (EGFP) under the control of the Moloney murine leukemia virus long terminal repeat (LTR) and has been previously described by Pollok et al. (2001). For generation of the GALV-pseudotyped vector, supernatant from an amphotrophic MFG-EGFP clone was used to infect the PG13 packaging line (American Type Culture Collection (ATCC), Manassas, Va.), and infected cells were isolated by single cell cloning. Individual clones were screened for titer by infecting 5 $\times$ 10⁵ human erythroleukemic cells (HEL) (ATCC) and determining the percent EGFP expression 48 hours after end-point dilution of supernatant. MFG-EGFP clone 5 has a titer of 0.5-1 $\times$ 10⁶ infectious units/ml and was used for experiments.

Retroviral Transduction of Endothelial Cells

[0143] Early passage (1-2) endothelial colony-derived endothelial cells were transduced with equivalent starting titers of MFG-EGFP supernatant. Six well non-tissue culture plates were coated with 5 μ g/cm² fibronectin CH-296 (Takara Shuzo, Otsu, Japan) for 2 hours at room temperature or overnight at 4° C. Plates were washed one time with PBS, and endothelial cells were plated at 5 $\times$ 10⁴ cells/cm² for transduction. Cells were infected with retrovirus supernatant diluted 1:1 with complete EGM-2 for 4 hours on 2 consecutive days with a change of complete EGM-2 media for overnight incubation. After the second round of infection, cells were harvested, counted and analyzed for EGFP expression by fluorescence cytometry.

Single Cell Assays

[0144] Early passage (1-4) endothelial colony-derived endothelial cells, transduced with the MFG-EGFP retrovi-

rus, were sorted by fluorescence cytometry for EGFP expression. A FACS Vantage Sorter (Becton Dickinson) was used (sort nozzle ≥ 100 microns at a sheath pressure of ≤ 9 pounds per square inch) to place one single endothelial cell expressing EGFP into each well of a 96 well flat bottom tissue culture plate pre-coated with type I collagen containing 200 μ l of complete EGM-2 media. Individual wells were examined under a fluorescence microscope at 50 $\times$ magnification to ensure that only one cell had been placed into each well. Cells were cultured at 37° C., 5% CO₂ in a humidified incubator. Media was changed every four days by removing 100 μ l and replacing it with 100 μ l of fresh complete EGM-2 media. At day 14, each well was examined for the growth of endothelial cells from the single plated cell. To quantitate the frequency of dividing single endothelial cells, the number of wells, which had 2 or more endothelial cells with a fluorescent microscope at 100 $\times$ magnification were counted. To enumerate the number of cells per well, the cells were counted by visual inspection with a fluorescent microscope at 100 $\times$ magnification (less than 50 cells per well), or the cells were trypsinized and counted them with a hemacytometer utilizing a trypan blue exclusion assay (more than 50 cells per well).

[0145] The long term proliferative and replating potential of endothelial cells derived from a single cell was determined. At day 14 after initiation of culture, individual wells containing greater than 50 cells were trypsinized, collected in 500 μ l of complete EGM-2 media and subcultured to a 24 well tissue culture dish coated with type I collagen. Four days after subculturing the cells, the media was aspirated and replaced with 500 μ l of fresh complete EGM-2 media. On day 7, wells were examined for colony growth or cell confluence by visual inspection with a fluorescent microscope at 50 $\times$ magnification. Cells were then trypsinized, counted, and subcultured in a 6 well tissue culture plate precoated with type I collagen. Following 7 days of culture in a six well plate, 10-12 wells, which contained confluent cell monolayers, for long-term cultures were selected under the conditions disclosed herein. For each sample, PDT and CPDL were calculated.

[0146] CD Markers: CD14 (lipopolysaccharide receptor), CD31 (platelet endothelial cell adhesion molecule), CD34 (sialomucin), CD45 (common leukocyte antigen), CD105 (endoglin), CD117 (c-Kit receptor), CD133 (prominin 1), CD141 (thrombomodulin), CD144 (vascular endothelial cadherin), CD146 (endothelial associated antigen, S-endo-1), flk-1 (fetal liver kinase-1, receptor for vascular endothelial growth factor 2).

Confocal Imaging of EPC

[0147] Passage 3-5 EPC were grown in a T 75 flask for four days using EBM-2 media with 10% added FBS. When cells reached confluence, media was aspirated, 5 ml of sterile PBS was added to the flask, and then aspirated, trypsin—EDTA was added and the flask was incubated for 5 min at 37° C. To quench the trypsin, 5 mL of EBM-2 media with 10% FBS was added and the released EPC were centrifuged at 350 $\times$ G for 10 min. The pelleted cells were washed with PBS and then resuspended in EBM-2 media with 10% FBS.

[0148] Glass chamber slides (4 chamber configuration; Corning) were coated with extracellular matrix proteins (e.g. collagen type 1 or 4, fibronectin, or vitronectin) over night

at 4° C. and then washed with sterile PBS in the morning. The PBS was aspirated and cells in EBM-2 media with 10% FBS were added at 50 cells per chamber and incubated at 37° C. in 5% CO₂ for 7 days.

[0149] EPC containing slides were washed twice with PBS and cells were fixed in acetone for 10 min at room temperature, washed twice with PBS, and blocked and permeabilized for 30 minutes with PBS, 3% nonfat dry milk, and 0.1% Triton X100. To highlight the plasma membrane of the cells, a primary antibody to CD146 conjugated to phycoerythrin (PE) was added (1 μ g/mL) to the fixed cells along with 1.5 mg/mL DAPI for nuclear staining. After a 30 minute incubation, cells were washed twice in PBS and examined for fluorescence using a Zeiss 510 confocal microscope. An ultraviolet laser (351/364 nm excitation) and a helium-neon laser (543 nm excitation) were used to excite the DAPI and PE-labeled cells through a 40 $\times$ water objective with the zoom kept on 0.7 $\times$ magnification. Images were captured in a single plane and displayed as monochromatic images for presentation. NIH Image software was used to quantify the nuclear and cytoplasmic diameters of cells from various EPC colony types.

TABLE 1

Enumeration of the number and time of appearance of endothelial progenitor cell colonies isolated from adult peripheral and umbilical cord blood mononuclear cells.					
Adult Peripheral Blood			Umbilical Cord Blood		
Donor	Number of Colonies/ 20 ml Blood	Day of First Colony	Donor	Number of Colonies/ 20 ml Blood	Day of First Colony
1	0.50	15	1	8.18	7
2	0.83	17	2	5.45	6
3	0.35	13	3	13.97	7
4	0.83	21	4	4.92	7
5	0.58	13	5	9.79	7
6	0.17	22	6	1.14	8
7	0.00	—	7	4.09	10
8	0.60	14	8	9.55	6
9	1.00	18	9	11.33	6
10	0.40	17	10	0.67	6
11	0.60	14	11	16.00	5
12	0.60	17	12	6.00	7
13	0.00	—	13	16.00	6
14	0.33	12			
15	1.33	11			
16	0.67	13			
17	0.00	—			
18	1.00	16			

Co-Culture of HPP-ECFC and CD34+ Cells Expands NOD-SCID Repopulating Cells.

[0150] Human CD34+ bone marrow cells, which have previously been shown to harbor marrow repopulating cells in NOD/SCID mice (SRCs) were isolated. Typically 0.5-1.0 $\times 10^6$ human marrow CD34+ cells are injected into NOD/SCID mice in order to achieve a level of human CD45+ chimerism of 5-50%. Initially only 9 $\times 10^3$ CD34+ cells were injected into NOD-SCID mice as a control on the day of harvest from human bone marrow. 9 $\times 10^3$ CD34+ cells were cultured in the presence of SCF, G-CSF, TPO, and Flt-3 for seven days. These are the growth factors currently used to maximally expand HSCs ex vivo. 9 $\times 10^3$ CD34+ cells were co-cultured with monolayers of cord blood HPP-ECFC

derived progeny in the absence of growth factors for seven days. Following seven days of culture, the cultured CD34+ cells were injected into NOD-SCID mice and the peripheral blood of transplanted mice was tested for the presence of human cells four weeks after transplantation. Co-culture of CD34+ cells with growth factors for 7 days increased the percentage of human cells detected in NOD-SCID mice 8 weeks after transplantation 10 fold compared to CD34+ cells injected shortly after isolation from human bone marrow. Despite injecting a very limited number of cells compared to prior studies, co-culture of CD34+ cells with cord blood HPP-ECFC-derived cells increased the percentage of human cells detected in NOD-SCID mice 8 weeks after transplantation 260 fold. Both human myeloid and lymphoid lineages were detected eight weeks after transplantation indicating that multilineage reconstitution of the hematopoietic system was achieved with CD34+ cells co-cultured with cord blood HPP-ECFC.

[0151] Starting with 2 T75 flasks of confluent monolayers of HPP-ECFC-derived cells, cells were first washed with Hanks balanced salt solution without calcium or magnesium (HBBS), then 1.5 mL of Trypsin EDTA (Gibco) was added to each flask for 1 minute. Next 8.5 mL of endothelial basal medium 2 (EBM2) (Cambrex) with 10% fetal bovine serum (FBS) (Hyclone), was added and suspended cells were collected and counted via Trypan blue exclusion on a hemacytometer.

[0152] HPP-ECFC- derived cells were plated at 3×10^5 cells/well onto collagen 1 precoated 6 well tissue culture plates (BD Biosciences). Cells were cultured with endothelial growth medium 2 (EGM2) (Cambrex) supplemented with 10% FBS and cultured overnight. The following morning the confluent cell monolayers were washed with EBM2+10% FBS twice and then co-cultured with 9,000 CD34+ CD38^{dim} Lin⁻ (CD4, 8, 11b, 14, 24, 31, 33, and glycophorin A) adult human bone marrow-derived cells collected by fluorescence activated cell sorting resuspended in 4 mL of EBM2+10% FBS+human megakaryocyte growth derived factor (MGDF) (100 ng/mL), granulocyte colony stimulating factor (G-CSF) (100 ng/mL), and stem cell factor (SCF) (100 ng/mL), and flt-3 ligand (100 ng/mL). Cells were cultured in 37° C. 5% CO₂ humidified incubator for 7 days without disturbance. In some cultures the CD34+ cells were co-cultured with the HPP-ECFC in EBM2+10% FBS and no added growth factors.

[0153] A 5 mL pipette was used to aspirate the nonadherent cells and media (4 mL) at the end of the 7 day co-culture. Wells were washed once with 2 mL phosphate buffered saline (PBS) and the PBS with nonadherent cells added to the original aspirate. To the same wells, 1 mL of cell dissociation buffer (Gibco) was added for 4 minutes at room temperature, and then the cell dissociation buffer and loosened cells were titrated in the well gently before aspiration and adding to the original aspirate. Finally, the HPP-ECFC monolayers were washed one final time with 2 mL of PBS and this solution with scant cells was added to the original aspirate. The final volume of media and cells was 9 mL.

[0154] The cell suspension was centrifuged at 1500 rpm (514×g) at room temperature for 10 minutes. The solution was removed and the cell pellet was dislodged mechanically then resuspended in 1/2-1 mL of EBM2+10% FBS. Cells were counted in Trypan blue on a hemacytometer. Recov-

ered cells were plated in progenitor assays or injected intravenously into NOD/SCID mice.

[0155] The method outlined above may be modified to provide a graft for a human transplant. In this instance, the HPP-ECFC progeny is plated in T75 flasks or in a perfusion chamber system to permit large numbers of CD34+ hematopoietic stem cells (autologous or allergenic human cord blood-, mobilized peripheral blood-, or marrow-derived) to be expanded in the presence of the cord blood HPP-ECFC. Systems are used that will permit the donor CD34+ cells to be cultured with the HPP-ECFC progeny without the cells directly touching and, thus, the donor CD34+ cells can be expanded, recovered, and transplanted into the human patient without the donor cells being "contaminated" with the cord blood HPP-ECFC progeny.

Cellularized Gel for In Vivo Implants

[0156] The gel mixture is made following standard protocols. Cells are apportioned to tubes. The gel mixture is added to cells. The cellularized gel mixture is apportioned to 12 well plates to warm the mixture (e.g., 37° C). The cell culture media is added to solidified gel for overnight culture. The cellularized gel is transferred to a desired site.

[0157] The gel mixture is kept cold to prevent gelation.

Volume Calculations

[0158] Since the gel material is highly viscous, the exact volume intended is not recovered (some material always sticks to the tubes), so if 4 gel implants are needed, a total volume of 2 mL gel is used. This is added to wells of a 12 well plate. For implantation, 1/2 of the well contents are used for a single implant. If 2 mL of gel (final) are needed, then 1.2 times the final amount is prepared to enable accurately retrieval of a viscous mixture from the tent tube (thus for 2 mL $\times 1.2 = 2.4$ mL. Additional gel material can also be prepared because some times bubbles hinder recovery of gel material from the tubes.

Calculation to Add All Reagents Necessary for Making 2 mL Cellularized Gel (for 4 Implants In Vivo).

[0159] EBM-2 (Cambrex, no supplements) w/10% serum and 1% antibiotics are needed. HEPES at a concentration of 1M (Cambrex 17-737E) is obtained. Sodium bicarbonate at 1.5 mg/ml (Sigma) is obtained. Rat Tail type 1 collagen at 3.88 mg/ml (Becton Dickinson) is obtained. Fibronectin (100 µg/ml; Chemicon, plasma fibronectin, FCO10-10 mg) is obtained. Fetal bovine serum (Hyclone SH30070.03) is obtained.

[0160] The pH is adjusted to 7.4 with 1N NaOH at 10 µl/ml collagen gel mixture. EBM-2-0.302 mL; FBS-0.440 mL; HEPES 0.110 mL; NaHCO₃ 0.088 mL; Collagen 1.700 mL; and fibronectin 0.440 mL are mixed. The total volume is 3.08 mL of gel constituents. All the constituents are kept on ice to prevent unwanted gelation.

[0161] The cells are divided and transferred to tubes. Generally between 1-2 million cells/mL gel are used. 1.2 times the final cell number needed is resuspended in (example if 1 million/mL wanted add 1.2 million) in 330 µl GM-2.

[0162] Cell volume occupies about 0.030 mL so the total cells in EGM-2 equals 0.0360 mL. The gel mixture (as generated above) is added to cells in the tubes. 840 µl of the

gel mix is added per tube, with 0.036 mL of cells in EGM-2 (volume=7.2 mL). The cellularized gel mixture is transferred to a plate to warm in an incubator.

[0163] 1 mL of cellularized gel mixture is recovered from the 1.2 mL in the tube and is added to one well of a 12-well plate. The cellularized gel is placed in 37 degree C. incubator for about 15-30 minutes to harden. The gel becomes opaque and white in appearance. The hardened gel is not clear. Growth media is added to solidified gel. 1 mL of EGM-2 is added per gel, by gently overlaying media onto gel. This assures adequate hydration during the overnight incubation.

[0164] Implantation

[0165] In a laminar flow hood, 7-12 week old NOD/SCID mice are anesthetized with 1/5% Isoflurane, and Nair is applied spot-wise bilaterally to the abdomen for—2 minutes. Nair is wiped away w/gauze, the skin cleaned with sterile PBS, then the sites of incision are saturated with povidine solution and allowed to dry. The skin is pinched away from the abdominal muscles and through a small incision, a pouch is formed by dissecting the skin and subcutaneous tissues from the muscle layer (procedure is repeated on other side of animal). One-half of each gel is then inserted into each pouch. The gel is positioned without tearing or mutilating the gel, and the skin is closed with suture. Mice are monitored daily for infection, change in diet, weight, and the like. Harvesting occurs 21-28 days post-implantation.

1. A method for forming a functional vasculature in vivo in a host, the method comprising:

- (a) culturing a plurality of isolated high proliferative-potential endothelial colony forming cells (HPP-ECFC) in a support material;
- (b) implanting the support material comprising the endothelial colony forming cells to a target site; and
- (c) forming the functional vasculature in vivo in the target site in the host.

2. The method of claim 1, wherein the support material is an artificial matrix.

3. The method of claim 2, wherein the matrix is a gel comprising one or more components selected from the group consisting of collagen, fibronectin, gelatin, laminin and any extracellular matrix constituent.

4. The method of claim 1, wherein the ECFCs are circulating ECFCs.

5. The method of claim 1, wherein the target site comprises an ischemic injury.

6. The method of claim 1, wherein the target site is selected from the group consisting of heart, lungs, kidney, liver, and pancreas.

7. The method of claim 1, wherein the host is a human.

8. The method of claim 1, wherein the target site requires vessel formation.

9. The method of claim 1, wherein the endothelial colony forming cells comprise a density of about 1-2 million cells per milliliter volume of implantation support material.

10. The method of claim 1, wherein the endothelial colony forming cells are cultured for about 12-48 hours prior to implantation.

11. The method of claim 1, wherein the support material is viscous.

12. The method of claim 1, wherein the support material provides three-dimensional cell growth.

13. The method of claim 1, wherein the support material comprising the cells is administered directly to the target site.

14. A method for forming functional blood vessels in vivo in a host, the method comprising:

- (a) culturing cord blood endothelial colony forming cells (ECFCs) in vitro;
- (b) administering the cultured cells to a target site; and
- (c) forming the vasculature in vivo in the host.

15. An implantable scaffold for treating an ischemic injury in a host, the scaffold comprising high proliferative-potential endothelial colony forming cells (HPP-ECFC), wherein the endothelial colony forming cells associate to form blood vessels in vivo.

16. The scaffold of claim 15 comprising a growth factor.

17. An engineered tissue comprising a biologically compatible support material and a plurality of cultured high proliferative-potential endothelial colony forming cells (HPP-ECFC) that initiate vessel formation.

18. A composition comprising an effective amount of high proliferative-potential endothelial colony forming cells (HPP-ECFC) culture on a biocompatible gel material.

19. The composition of claim 19 comprises one or more components of an extra cellular matrix.

20. The composition of claim 19, wherein the ECFCs are cultured on a growth medium for endothelial cells.

* * * * *