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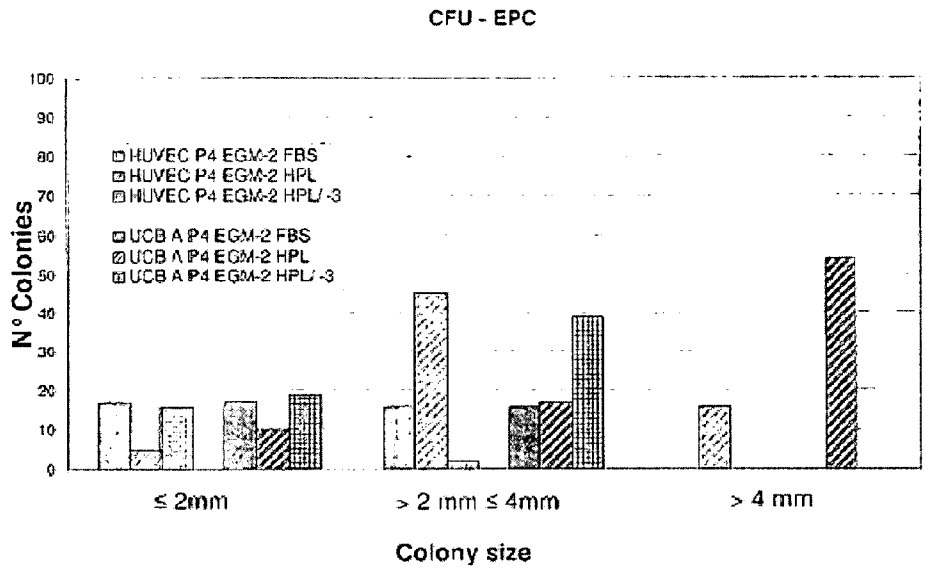
(54) **Title:** METHOD TO STUDY PROLIFERATION OF ENDOTHELIAL PROGENITOR CELLS AND THE POTENTIAL INFLUENCE OF COMPOUNDS ON THEIR PROLIFERATION BEHAVIOUR

Figure 5



(57) **Abstract:** The present invention provides a simple and rapid method for determining colony forming units (CFU) of endothelial progenitor cells (EPC). Furthermore, a method for identifying compounds exerting an effect on those CFU of EPC is provided. The present invention also provides medium for culturing endothelial cells which is comprised of EGM-2, hydrocortisone and VEGF as well as human platelet lysate (HPL) as supplement.

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Method to study proliferation of endothelial progenitor cells and the potential influence of compounds on their proliferation behaviour

SUBJECT OF THE INVENTION

The present invention provides a method for determining the colony forming units (CFU) of endothelial progenitor cells (EPC). To analyze the influence of compounds on the proliferation behaviour of EPC, the present invention also provides a method for analyzing said influence by comparing the CFU of EPC under conditions exposed to test compounds and control conditions. This also comprises the analysis of the influence of different conditions on the CFU of EPC by comparison to standard conditions. The present invention further provides a specific medium for culturing endothelial cells.

BACKGROUND OF THE INVENTION

Colony assay methods have been widely used for studying progenitor cell presence in hematopoietic tissues. The assessed progenitor cells are not necessarily of hematopoietic origin, because a large variety of cells is present in the hematopoietic tissues of the bone marrow, the thymus and the spleen. This comprises erythrocytes and leucocytes as types of hematopoietic cells (and the related mast cells), but also fat cells, fibroblasts and endothelial cells as well as their corresponding progenitor cells, which are able to form macroscopic colonies and are therefore also generally called colony-forming cells (CFC).

A tissue culture method allowing for the clone counting of mammalian cells has first been established in 1956 (Puck T. and Marcus P. (1956) J. Exp. Med. 103(5): 653-666). In their publication, the authors describe an *in vitro* culture method to grow single mammalian HeLa cells into macroscopic colonies. Using this assay, researchers were enabled to grow, count and analyze colonies of bone marrow-derived cells, but there was no possibility to differentiate between the different cell types present in bone marrow.

As a method comparable to such an *in vitro* techniques, the *in vivo* spleen colony

technique has been introduced in 1961 (Till J. and McCulloch E. (1961) Radiat. Res. 14:653-666). With this technique, it became possible to analyze the colony formation capacity of injected cells, which formed colonies in the spleen of mice. Those proliferating cells, from which the colonies derived from, were called colony forming units in the spleen (CFU-S). Prior to the histological analysis of the spleen, the mice had first been exposed to supralethal total-body irradiation and then a cell suspension of the bone marrow had been intraveniously injected. Again, it was only possible to analyze the CFU of the total bone marrow cells without any further distinction. The general need for such assays (*in vivo* or *in vitro*) was expressed by Till and McCulloch as follows: "The variability found in the numbers of CFU in different marrow suspensions indicates the need for such a measure."

From 1965 on, it was possible to discriminate between certain cell types of hematopoietic tissues. Pluznik and Sachs described a method for the detection of precursor cells of mast cells from hematopoietic organs with the capacity to form colonies in an agar tissue culture, which was also used to detect precursor cells committed to granulopoiesis and macrophage formation, the so called CFU-GM (Pluznik D. and Sachs L. (1965) J. Cell Physiol. 66(3): 319-324; Bradley T. and Metcalf D. (1966) Aust.J. exp. Biol. Med. Sci. 44: 287-299). Fibroblast precursors existing within the hematopoietic system also have been evaluated with a colony assays method (CFU-F), introduced by Friedenstein in 1974 (Friedenstein A. et al. (1974) Exp. Hematol. 2(2): 83-92).

CFC assays for hematopoietic cells are routinely used standard tests to determine the functional capacity and quality of hematopoietic stem cells (HSC) in the case of clinical HSC transplantation. Also, longterm culturing of initiating cells (LTC-IC) derived from human bone marrow enables researchers to investigate these very primitive HSC (Petzer et al. (1996) J. Exp. Med. 183(6): 2551-2558).
As mentioned above, endothelial cells (EC) and endothelial progenitor cells (EPC)

are among hematopoietic cells, fat cells and fibroblasts (and the related mast cells) also known to reside in the hematopoietc tissues. There is growing evidence that the EPC contribute to tissue vascularization and first clinical studies indeed have shown that introduction of bone marrow-derived endothelial and hematopoietic progenitor cells can restore tissue vascularitzation after ischemic events in limbs, retina and myocardium (for a review see Rafii S. and Lyden D. (2003) Nat. Med. 9(6): 702-712). There is hope to use stem cells and progenitor cells for organ regeneration in regenerative medicine, but, of course, cells used for such applications need to be characterized with respect to their phenotypical and morphological properties on one side and their proliferative behaviour on the other side.

EPC are characterized on a phenotypical and morphological level to a rather good extent. For example, it has been shown that statins induce EPC differentiation from mononuclear cells and therefore lead to an increase in total numbers of EPC (Dimmeler et al. (2001) J. Clin. Invest. 108(3): 391-397). On a molecular level, a subset of circulating human cells expressing the surface marker CD34 was shown to also express VEGFR-2 and AC133. Those $CD34^+$, $VEGFR\text{-}2^+$ and $AC133^+$ circulating cells seem to represent functional endothelial precursor cells which may play a role in neo-angiogenesis (Peichev M. et al. (2000) Blood 95(3): 952-958). In a mouse model, the infusion of *in vitro* differentiated EPC, which derived from mononuclear cells (MNC) of the spleen, into mice with strong defects in the endothelium-dependent vasodilation led to a significant improvement of the defects. Those differentiated cells were shown to be Dil-Ac-LDL/lectine-positive (Wassmann S. et al. (2006) Circ Res. 99:74-83).

To analyze the capacity of EPC to form colonies, only one assay has been described so far (Ingram et al. (2004) Blood 104(9): 2752-2760). The purpose of this assay was to determine if there is a hierarchy among human EPC. Using this assay, the authors were able to show that EPC form colonies *in vitro*, when deposited at the single-cell

level. The protocol for this assay consists of the following main steps (for further detail see Ingram et al. (2004) Blood 104(9): 2752-2760): At first, MGF-EGFP supernatant with a titer of 0.5-1 x 10^6 infectious units/ml is needed for the transduction of the EPC. To obtain this supernatant, appropriate cells are transfected with a retroviral vector, which contains the coding sequence for EGFP under the control of the Moloney murine leukemia long terminal repeat. Viral supernatant produced by those cells is then assayed for its infectious titer after two to three days of incubation. Following the determination of the titer, the EPC are then infected with this retrovirus containing supernatant for four hours on two consecutive days. Using a FACS machine, the cells are in a further step sorted by fluorescence cytometry using the expression of exogenous EGFP and, with the help of a cell sorter, cells positive for EGFP expression are in the next step placed as single cells in the wells of a precoated 96-well tissue culture plate. Individual cells are examined under a fluorescence microscope to ensure that only one cell has been placed into each well. After 14 days of incubation, the colony growth is scored and large colonies are further subcultured for another seven days and, if they still show growth after this incubation period, again subcultured for another seven days. EPC with high proliferative potential (HPP) are defined as large colonies that form secondary and tertiary colonies after replating as described.

In summary, the assay for analysis of the proliferation of EPC described above uses a rather time-consuming and elaborate protocol. It is for example necessary to provide retrovirus containing supernatant to infect the cells with vector coding for EGFP. EPC analyzed following this protocol are therefore always modified in such a way, that they contain retroviral DNA and express exogenous EGFP for identification and sorting reasons. Furthermore, technical equipment like a FACS machine coupled to a cell sorter and a fluorescence microscope are required to proceed according to the protocol. Of course, persons using this assay to determine the CFU of EPC need to be experienced with the technical equipment just mentioned.

Therefore, there is a need for a simple method, which is easy to handle and can be used by a person not necessarily having a strong background in instrumental setups. The method should furthermore be as cheap as possible and adapted for a rapid determination of the proliferation of EPC.

OBJECT AND SUMMARY OF THE INVENTION

It is an object of the present invention to provide a simple and rapid method for determining the colony forming units (CFU) of endothelial progenitor cells (EPC) which can be used, for example, to judge the quality of EPC-preparations applied in regenerative medicine or clinical transplantations.

It is yet another object of the present invention to provide a method for analyzing the influence of compounds and/or test conditions on the colony forming unit (CFU) capacity of endothelial progenitor cells (EPC). Such a method can be used to screen test compounds for potential positive or harmful effects on EPC proliferation.

It is a further object of the present invention to provide a simplified cell culture medium for culturing endothelial cells and, of course, also endothelial progenitor cells.

These and further objects of the invention, as will become apparent from the ensuing description, are attained by the subject-matter of the independent claims. Further embodiments of the invention are defined by the dependent claims.

According to one aspect of the invention, a method of determining the colony forming units (CFU) of endothelial progenitor cells (EPC) is provided, comprising the following steps:

a) Seeding starter cells in an appropriate medium at such a density, that contact inhibition cannot take place;

b) Incubating cells for several days; and

c) Identifying colonies.

According to another aspect of the invention, a method of identifying compounds and/or conditions exerting an effect on the CFU of EPC is provided, comprising the following steps:

a) Seeding starter cells in an appropriate medium at such a density, that contact inhibition cannot take place;

b) Contacting cells with one or several test compound(s) and/or test conditions;

c) Incubating cells for several days;

d) Identifying colonies; and

e) Comparing with a control.

In preferred embodiments of the methods of the present invention, colonies are identified by staining of the colonies and/or counting of the colonies.

In a preferred embodiment of the methods of the present invention, starter cells comprise human umbilical vein endothelial cells (HUVEC, commercially available from Cambrex, USA), human microvascular vein endothelial cells (HMVEC, commercially available from Cambrex, USA),endothelial cells prepared from umbilical cord blood (UCB-EC, preparation according to example 1) and EPC derived from any mammalian solid organ or vasculature such as the bone marrow or the lung.

In another preferred embodiment of the methods of the present invention, native, not modified starter cells are used.

In another preferred embodiment of the methods of the present invention, cells are used, which express exogenous protein(s) excluding any protein which can be used as marker for identification and sorting purposes.

In a further preferred embodiment of the methods of the invention, the medium chosen for culturing of the cells comprises EGM-2, hydrocortisone, VEGF, ascorbic acid and heparin and is supplemented with FBS.

In yet another embodiment of the methods of the invention, the medium chosen for culturing of the cells comprises EGM-2, hydrocortisone, VEGF, ascorbic acid and heparin and is supplemented with human platelet lysate (HPL, prepared from platelet rich plasma) instead of FBS.

In a further aspect of the methods of the present invention, the medium chosen for culturing of the cells comprises EGM-2, hydrocortisone, VEGF, ascorbic acid and heparin, is supplemented with FBS or HPL and additionally comprises small amounts of FBS.

In yet another aspect of the methods of the present invention, the medium chosen for culturing of the cells comprises EGM-2, hydrocortisone, VEGF, ascorbic acid, heparin and small amounts of FBS, is supplemented with FBS or HPL and also comprises VEGF, human fibroblast growth factor B and the R3-insulin like growth factor 1.

In a further embodiment of the methods of the invention, the cells are seeded at a density of 1 cell/cm^2 in a 150 cm^2 or a 55 cm^2 culture dish to ensure, that the cells are proliferating without any contact inhibition.

In yet another embodiment of the methods of the invention, the cells are incubated for 12 days with a medium change every third day.

In a further aspect of the methods of the present invention, the cells and colonies are stained with Harris` Hematoxylin staining.

In yet another aspect of the methods of the invention, a colony contains more than 50 cells and is defined according to its size as derived from a regular CFU or a CFU with high proliferative potential (HPP).

In another preferred embodiment of the methods of the invention, above mentioned HPP-CFU is defined as having a colony size > 2.0 mm and a regular CFU is defined as having a colony size <= 2.0 mm if EGM-2 medium containing FBS as supplement or EGM-2 medium containing HPL as supplement and lacking the three growth factors VEGF, human fibroblast growth factor B and the R3-insulin like growth factor 1 is used.

In still another embodiment of the methods of the invention, above mentioned HPP-CFU is defined as having a colony size > 4.0 mm and a regular CFU is defined as having a colony size <= 4.0 mm if EGM-2 medium supplemented with HPL and comprising hydrocortisone, VEGF, ascorbic acid, heparin, optionally small amounts of FBS, VEGF, human fibroblast growth factor B and the R3-insulin like growth factor 1 is used.

In yet a further aspect of the method for identifying compounds and/or analyzing the influence of test conditions of the present invention, a control is defined as an assay under identical conditions, wherein the appropriate medium does not contain one or several test compound(s) or an assay under standard conditions.

In a further preferred embodiment of the method for identifying compounds/conditions of the present invention, a test compound/condition is defined

as a positive regulator of the CFU-EPC when it is increasing the number of CFU compared to a control.

In yet another preferred embodiment of the method for identifying compounds/conditions of the present invention, a test compound/condition is defined as a negative regulator of the CFU-EPC when it is decreasing the number of CFU compared to a control.

In another aspect of the method for identifying compounds of the present invention, the test compound or compounds is/are selected from the group of test compounds comprising small molecules derived from small molecule libraries, growth factors and compounds of pharmaceutical interest.

According to another aspect of the invention, a cell culture medium is provided which comprises EGM-2, hydrocortisone, VEGF, ascorbic acid and heparin and is supplemented with HPL.

In a further aspect of the invention, a cell culture medium is provided which comprises EGM-2, hydrocortisone, VEGF, ascorbic acid, heparin, small amounts of FBS and is supplemented with HPL.

In a further embodiment of the method of the invention, the provided cell culture medium is used to culture endothelial cells.

DESCRIPTION OF THE DRAWINGS

Fig. 1A shows early outgrowth of endothelial cells in primary culture from umbilical cord blood (UCB-EC) after five days of culture.

Fig. 1B shows a confluent cobblestone-like monolayer of late outgrowth UCB-EC at passage 3 and 23 days of culture.

Fig. 2 depicts phenotypic FACS-profiling of UCB-EC, HUVEC an HMVEC analyzing the expression of CD31, CD90, CD13, CD29, CD73, CD105, CD146, HLA-AB and HLA-DR as well as binding of BS-1 lectin.

Fig. 3 shows colonies of CFU-EPC assays using UCB-EPC (passage 4). The photographs were taken after 12 days of incubation. UCB-EPC depicted on the left side were incubated with HPL EGM-2 medium (see appendix), whereas the UCB-EC depicted on the right side were incubated with conventional EGM-2 medium (supplemented with FBS instead of HPL, see appendix).

Fig. 4 shows the number of total colonies and photographs of culture dishes after staining. The fold increase of cells after an incubation period of 12 days is also indicated. Cells (HUVEC or UCB-EC) were either incubated with HPL EGM-2 medium (marked as _HPL, see appendix) or HPL-3 EGM-2 medium, which lacks the growth factors VEGF, human fibroblast growth factor B and the R3-insulin like growth factor 1 (marked as _HPL/-3).

Fig. 5 depicts the numbers of colonies of various colony sizes as indicated. HUVEC or UBC-EC (each passage 4) incubated either with conventional EGM-2 (marked FBS), HPL EGM-2 (marked HPL) or HPL-3 EGM-2 (marked HPL/-3) medium were tested in the CFU-EPC assays.

Figures 6 – 10 show standardized quantifications using the CFU-EPC assay as described in the present invention with conventional EGM-2 medium. Absolute colony counts (upper diagram), the number of HPP CFU-EPC (middle diagram) and the percentage of HPP CFU-EPC (bottom) are given. Erythropoietin (EPO) was added to the medium in the amounts indicated underneath the bars. The following cells were used for the assay:

Fig. 6: HUVEC (passage 3)

Fig. 7: HMVEC (passage 2)

Fig. 8 : UCB-EC (passage 3)

Fig. 9 : UCB-EC (passage 4)

Fig. 10 : UCB-EC (passage 5)

DETAILED DESCRIPTION OF EXEMPLARY EMBODIMENTS

While describing in detail exemplary embodiments of the present invention, definitions important for understanding the present invention are also given.

The term "colony forming units" or "CFU" defines the capacity of cells, in the context of the present invention preferably progenitor cells, to form macroscopic colonies while proliferating.

The term "proliferation of cells" is intended to mean the multiplication of cells thereby leading to an increase in the cell number. The proliferation of cells may be detected by any suitable method. The easiest way to measure proliferation is to seed the cells in a specific, predetermined density and to count the cell number at different time points after seeding. Another way of measuring the proliferation of cells is a [^{3}H]-thymidine incorporation assay which involves the addition of [^{3}H]-thymidine to the cells, incubating them for a specific time, lysing the cells and counting the incorporation in a scintillation counter. Commercially available kits like the tetrazolium assay (MTT, Sigma) may also be used for measuring proliferation.

In the context of the present invention, a "colony" is defined as consisting of preferably at least 200, more preferably of at least 100 and most preferably of at least 50 cells after incubation and proliferation of the single CFU. This is dependent on the size of the endothelial cells used in the assay. In the example section of the present invention, a colony is defined as consisting of at least 50 cells for the following cells: HUVEC (human umbilical vein endothelial cells, commercially available from Cambrex, USA), HMVEC (human microvascular vein endothelial cells, commercially available from Cambrex, USA) and endothelial cells prepared from umbilical cord blood (UCB-EC, preparation according to example 1.

"Progenitor cells" are early descendants of stem cells which may lose their ability of self-renewal. The progenitor cells are able to differentiate to different cell types within one germ layer, but in contrast to stem cells they cannot differentiate to cells of a different germ layer. There are three different germ layers, i.e. endoderm, ectoderm and mesoderm which are formed by gastrulation. The endoderm is the internal cell layer of the embryo from which the lung, digestive tract, bladder and urethra are formed. The ectoderm is the surface layer of the embryo that develops into the epidermis, skin, nerves, hair, etc. The mesoderm is the middle cell layer of the embryo from which the connective tissue, muscles, cartilage, bone, lymphoid tissues, etc. are formed.

Within the context of the present invention, the progenitor cells used are preferably endothelial progenitor cells from different cell preparations, most preferably UCB-EC, HUVEC and HMVEC. It is also preferred to use endothelial progenitor cells derived from any mammalian solid organ or vasculature, most preferably cells derived from the bone marrow or the lung.

The term "cell culture" refers to the maintenance and propagation of cells and preferably animal (including human-derived cells) *in vitro*. The cells may include progenitor cells as defined above.

According to the methods of the present invention, the cells are either used in a native, non modified way or are modified in such a way, that the expression of proteins, which can be used as markers for identification and sorting purposes is excluded.

More precisely, the term" native cells" means that the cells are not manipulated by any method to either express any exogenous gene or to contain exogenous DNA or RNA or the like, which may in any way influence the regulation of gene expression in the cells.

Alternatively, the cells may be transduced or transfected with DNA or RNA or manipulated in any other way, which may lead to expression of exogenous genes or silencing of endogenous genes or to any other effect on the regulation of gene expression, as long as the cells are not manipulated in such a way, that they express any protein, which is suitable as marker for the identification and sorting of cells using for example a FACS machine. This comprises for example GFP, EGFP, YFP, CFP and so on.

For the methods of the present invention, the cells are seeded in cell culture dishes at such a density, that contact inhibition cannot take place.

This means, that trypsinized and washed cells, dissolved in a defined volume of appropriate medium, are counted by any suitable method and then either concentrated or further diluted in appropriate medium to reach a certain density of cells defined for example as the number of cells per ml of medium. If the density of

cells has been determined, it is in a next step possible to add a defined volume of medium containing this defined amount of cells to an empty cell culture dish capable of absorbing a defined volume of medium.

The term "contact inhibition" means, that cells inhibit their cell divisions and therefore stop growing if they are in too dense proximity to each other. An area of 1 cm^2 per cell as used in the methods of the present invention is by far enough to exclude this phenomenon arising during proliferation. Thus, seeding endothelial cells at a density of 1 cell / cm^2 is the most preferred density of seeding the cells according to the present invention without the occurrence of contact inhibition. Depending on the cells, a density of 2 to 10 cells / cm^2 is also preferred.

The most preferred embodiment for culturing the cells is in any standard incubator suitable for growing cell culture cells, wherein cells are cultured at 37°C in 5% CO_2 in humidified atmosphere, also referred to as standard conditions known to the skilled person in the art in contrast to any test conditions.

One way of identifying colonies is to directly identify them on the cell culture dish. Alternatively, colonies can be identified by staining and/or counting of cells. Cells grown on a cell culture dish can be stained according to the methods of the present invention by any known and suitable protocol. This comprises staining of the cells for example with Harris Hematoxylin staining or tryptophan blue-solution with staining by Harris Hematoxylin being the most preferred way. If required, the cells must be fixed prior to staining, for example by cold Aceton/Methanol as described in the example section.

Directly identified or stained cells/colonies can be counted and colony sizes measured by visual inspection or with an analyzer and the corresponding software or by any other method suitable for determining the number of cells/colonies and the sizes of colonies. Most preferred is the use of a standard automatic analyzer, for

example the use of a ChemiDoc™ XRS Universal Hood II or an AID BacSpot™ System.

Test compounds, which can be added to the medium according to one embodiment of the present invention for determining a possible effect on the proliferation of endothelial progenitor cells can be selected from a large variety of molecules. This comprises molecules derived from libraries of small molecules, pharmaceuticals, molecules isolated from or derived from tissues like growth factors and so on. Most preferred compounds are neutralizing antibodies against growth factors or any other potential therapeutically active antibodies.

"Test Conditions" as mentioned above are conditions, which differ in any aspect from the standard conditions known to the person skilled in the art as defined above . Because the differing parameter(s) is/are known, it is possible to evaluate the influence of the specific parameter(s) by comparing the results to results obtained under standard conditions. One preferred parameter used for such studies is the oxygen concentration in the atmosphere used for culturing the cells. In most preferred embodiments, this comprises air oxygen conditions and low oxygen conditions (<= 3% O_2).

"Cell culture medium" ("medium") is used for the maintenance of cells in culture *in vitro*. For some cell types, the medium may also be sufficient to support the proliferation of the cells in culture. A medium according to the present invention provides nutrients such as energy sources, amino acids and anorganic ions. Additionally, it may contain a dye like phenol red, sodium pyruvate, several vitamins, free fatty acids and trace elements. In the context of the present invention, a preferred medium is a medium suitable for culturing endothelial cells (for example EGM-2 [endothelial cell medium] commercially available from Cambrex, USA).

The term "cell culture medium supplement" within the meaning of the present invention refers to a medium additive which is added to the medium to stimulate the proliferation of the cells. Usually this supplement will contain one or more growth factors which are responsible for the stimulation of proliferation.

In the context of the present invention, it is preferred to either use foetal bovine serum (FBS, commercially available from Hyclone, USA) or human platelet lysate (HPL) as cell culture medium supplement (Schallmoser et al. (2007) Transfusion, in press; Doucet et al. (2005) Journal of Cellular Physiology 205(2):228-236).

If HPL is used as cell culture medium supplement, it is preferred to use plasma-free HPL which is prepared according to a method comprising the following steps:

a) preparing platelet-rich plasma;
b) removing the plasma; and
c) lysing the platelets.

It is further preferred to prepare the platelet-rich plasma used for the production of HPL from buffy coat units. Furthermore, in another preferred aspect of the present invention concerning the use of HPL, recombinant albumin may be added to the culture supplement.

The term "supplement" is not intended to comprise medium additives which are added to the medium for the purpose of freezing the cells.

In addition to the cell culture medium supplement of the present invention, other additives such as additional specific growth factors (see below), hormones, glutamine, ribonucleotides, desoxyribonucleotides, antioxidants and antibiotics may be added to the medium, but do not fall into the category of supplements as defined above. More specifically, this comprises hormones such as hydrocortisone,

antioxidizing agents such as ascorbic acid, antibiotics such as gentamycin and antifungal substances such as amphotericin, which all may be added. Heparin may also be additionally added to the medium to increase the uptake of certain growth factors such as VEGF.

The term "growth factor" is intended to comprise proteins which stimulate proliferation of cells by binding to a specific receptor. Usually, growth factors only act on specific cell types which express the respective receptor. Examples of growth factors are epidermal growth factor (EGF), nerve growth factor (NGF), platelet derived growth factor (PDGF), fibroblast growth factor B (FGF), vascular endothelial growth factor (VEGF), bone morphogenetic proteins (BMP), colony stimulating factors (CSF), R3-insulin like growth factor 1 etc.
Medium according to the present invention comprises cell culture medium as defined above, preferably EGM-2 (commercially available from Cambrex, USA). Further additives added to the medium typically comprise at least hydrocortisone, VEGF, EGF, FGF, R3-insulin like growth factor 1 and heparin. Additional compounds which can be further added to the medium comprise antioxidants, antibiotics and anti-fungal agents.

A medium which can be used in the methods of the invention seems to require at least EGM-2, VEGF, hydrocortisone and FBS or HPL as supplement.

In yet further preferred alternatives, the additional minimal additives comprise at least hydrocortisone, 2% FBS, VEGF, EGF, FGF and R3-insulin like growth factor 1 or alternatively the minimal additional additives comprise at least hydrocortisone and VEGF.

As supplement as defined above, either FBS is added to the medium to a final concentration of approximately 1-20% v/v, preferably of approximately 5-18% v/v,

more preferably of approximately 8-12% v/v and most preferably of approximately 10% v/v.

In a preferred alternative, HPL instead of FBS is added to the medium to a final concentration of approximately 1-15% v/v, preferably of approximately 4-11% v/v, more preferably of approximately 6-9% v/v and most preferably of approximately 7,0% v/v. HPL is preferably a plasma-free HPL.

All the aforementioned type of media can comprise additional additives. Typically, the medium will additionally comprise an antioxidizing agent such as ascorbic acid, an antibiotic agent such as gentamycin, an antifungal agent such as amphotericin and an agent increasing the uptake of certain growth factors such as heparin.

In some embodiments, one may add also up to 2% FBS.

Therefore, according to the methods of the present invention, a suitable cell culture medium is comprised of EGM-2, hydrocortisone, optionally 2% FBS, VEGF, ascorbic acid, heparin, gentamycin, amphotericin, EGF, FGF and R3-insulin like growth factor 1 and 10% v/v FBS as supplement.

In an alternative embodiment, a suitable cell culture medium is comprised of EGM-2, hydrocortisone, optionally 2% FBS, VEGF, ascorbic acid, heparin, gentamycin, amphotericin, EGF, FGF and R3-insulin like growth factor 1 and 2% v/v FBS as supplement.

Yet another particularly suitable medium according to the methods of the present invention is medium comprised of EGM-2, hydrocortisone, optionally 2% FBS, VEGF, ascorbic acid, heparin, gentamycin, amphotericin, EGF, FGF and R3-insulin like growth factor 1 and 7.5% v/v HPL as supplement.

One of the most preferred embodiment of the present invention is cell culture medium comprising EGM-2, hydrocortisone, VEGF, ascorbic acid and heparin, supplemented with HPL to a final concentration of approximately 7.5% v/v. In a further preferred embodiment of the present invention, such cell culture medium comprises small additional amounts of FBS, most preferably approximately 2%.

It was surprisingly found, that medium comprising EGM-2, hydrocortisone, VEGF, ascorbic acid, heparin, 7,5% v/v HPL as supplement and, optionally, additionally 2% FBS, can be used as an alternative to medium comprising EGM-2, hydrocortisone, optionally 2% FBS, VEGF, ascorbic acid, heparin, gentamycin, amphotericin, EGF, FGF and R3-insulin like growth factor 1 and 10% v/v FBS as supplement.

The term "approximately" describes a deviation of the indicated value by at least 10 %, preferably by at least 5 % and most preferably by at least 1 %.

The term "HPP-CFU" describes CFU of endothelial cells with a high proliferative potential. This means in the context of the present invention, that there are some CFU among the total CFU, which exhibit a particular high proliferative rate and are thus forming colonies of larger size. Of course, the term "larger size" implicates a reference size of a colony, which is defined according to the present invention depending on the cell culture medium, which is used for culturing of the cells.

In a preferred CFU-EPC assay of the present invention, a HPP-CFU is defined as having a colony size larger than 2.0 mm if a medium comprising EGM-2, hydrocortisone, VEGF, EGF, FGF, the R3-insulin like growth factor 1 containing FBS as supplement or medium comprising EGM-2, hydrocortisone, VEGF containing HPL as supplement and lacking the three growth factors VEGF, FGF and the R3-insulin like growth factor 1 is used.

Using medium comprising EGM-2 (which may contain 2% FBS), hydrocortisone, VEGF and HPL as supplement as well as the three growth factors VEGF, FGF and the R3-insulin like growth factor 1, a HPP-CFU is defined in an alternative CFU-EPC assay, which is also preferred, as having a colony size larger than 4.0 mm. In both cases, a regular CFU is defined as having a colony size smaller than or equivalent to the sizes just mentioned (either smaller than or equivalent to 2.0 mm resp. 4.0 mm).

As mentioned above, a possible effect of one or several test compound(s) and/or test conditions on the proliferation of endothelial progenitor cells can be determined according to one method of the present invention. To be able to determine an effect of one or several test compound(s, which have been added to the medium as described above, the CFU-EPC need to be compared to a suitable control. In an analogous way, the influence of test conditions as described above can be analyzed by the comparison to standard conditions mentioned above.

The term "suitable control" in this context means, that an assay under identical conditions but without adding one or several test compound(s) to the medium is performed in parallel to the assay, wherein the cells are exposed to one or several compound(s). The term "in parallel" refers to the whole protocol of the assay including sizes of cell culture dishes, incubation times and media changes.

Depending on the comparison of the numbers of CFU-EPC, one or several test compound(s) and/or test conditions might have a positive or negative influence on the CFU-EPC. If the addition of one or several test compound(s) and/or test conditions leads to a significant increase in the numbers of CFU-EPC, the compound(s)/conditions show(s) a positive effect on the proliferation of EPC *in vitro*. Accordingly, the addition of one or several test compound(s)/certain conditions

leading to a significant decrease in the numbers of CFU-EPC, the compound(s) show(s) a negative effect on the proliferation of EPC *in vitro*.

A significant increase according to the present invention means that the test compound increases the CFU-EPC numbers by at least 3%, at least 5% or at least 10%. Preferably, the number is increased by at least 25%, 50% or 75%. More preferably, the number of CFU-EPC is increased by a factor of at least 2, 5 or 10.

A significant decrease according to the present invention means that the test compound reduces the CFU-EPC numbers by at least 3%, at least 5% or at least 10%. Preferably, the number is reduced by at least 15%, by at least 25% or at least 50%. More preferably, the number of CFU-EPC is reduced by at least 75%, 80% or 95%.

All numbers refer to a comparison of test and control assays each being performed in triplicates.

The present invention provides a cheap, simple and rapid alternative method for determining the CFU of EPC as well as a method for determining a potential effect of one or several test compound(s) and/or test conditions on the proliferation behaviour of EPC. Furthermore, the invention is based on the surprising finding that EGM-2 cell culture medium with hydrocortisone, VEGF, ascorbic acid, heparin and HPL as supplement but lacking the three growth factors EGF, FGF and R3-insulin like growth factor 1, can be used as an alternative to the same cell culture medium, but comprising FBS as supplement and the three growth factors mentioned above for culturing endothelial cells.

The invention is further illustrated by the following examples, which should not be construed as limiting. The contents of all references, patent applications, patents,

published patent applications, tables and appendices cited throughout this application are hereby incorporated by reference.

EXAMPLES

Example 1: Preparation of test endothelial cells derived from umbilical cord blood (UCB-EC)

It was the purpose of this example to provide human endothelial cells comprising endothelial precursor cells (EPC) as well as EPC undergoing differentiation. Following the preparation, the cells were characterized (example 2) and the method of the present invention (example 3) was used to determine the colony forming units (CFU) of the UCB-EPC. To compare the results to the CFU of other endothelial cells from different preparations and tissue origin and to determine whether those endothelial cells can, in principle, also be used in CFU-EPC assays of the present invention, commercially available human microvascular and umbilical vein endothelial cells were also characterized and tested (HMVEC and HUVEC, both from Cambrex, see the following examples).

To obtain functional endothelial precursor cells from circulating blood, umbilical cord blood (UCB)-derived EC were generated by culturing UCB-mononuclear cells according to previously published methods (Ingram D. et al. (2004) Blood 104(9):2752-2760). In brief, cord blood was collected in 50 ml vials containing 5000 I.U. heparin. After density gradient centrifugation, mononuclear cells were seeded into coated six-well plates in conventional EGM-2 medium (see appendix) at a density of 1-3x10^7/cm^2. Cells were cultured at 37°C in 5% CO_2 in humidified atmosphere and medium change was performed daily for seven days and afterwards every second day. First cobblestone-like colonies appeared between five and eight days (figure 1). At confluency, EPC were transferred to new coated dishes and after

passage 1 culture conditions were changed to non-coated culture flasks and conventional low serum EGM-2 medium (see appendix for medium and figure 1 for cells). UCB-EC were then cryopreserved for further use.

Example 2: Multiparameter flow cytometry to analyze surface markers of UCB-EC, HMVEC and HUVEC

It was the purpose of this example to show that all three types of endothelial cells (UCB-EC, HMVEC and HUVEC) used in the CFU-EPC assays are indeed of endothelial origin and express identical surface markers.

The surface marker expression of EC was analyzed with a four colour FACS-Calibur instrument equipped with a 488 nm argon ion laser and a 635 nm red diode laser (Becton Dickinson). After trypsinizing, cells were washed, blocked with sheep serum and labelled for 25 minutes at 4°C at concentrations according to individual titration with BS-1 lectin and monoclonal antibodies against HLA-AB (Harlan Sera-Lab), HLA-DR, CD13, CD14, CD29, CD31, CD45, CD56, CD73(BD), CD90, CD105 (Caltag Laboratories), CD117, CD123, CD133 (Miltenyi) and CD146 (clone P1H12, Chemicon). Appropriate isotype matched antibodies were used as negative controls (BD). Four colour measurements were performed and data from at minimum 10,000 viable propidium iodine excluding cells were stored. List mode files were analyzed with CellQuest Pro and Paint-A-Gate Pro Software (BD).

Figure 2 indicates that all three cell lines express or lack the same surface markers. The markers show a distribution typical for endothelial cells and it was therefore confirmed, that all three cell lines used in the tests of the following examples were indeed endothelial cells.

Example 3: Determination of colony forming units (CFU) of endothelial progenitor cells (EPC) in UCB-EC, HMVEC and HUVEC

It was the purpose of this example to determine the presence of EPC in the three cell lines mentioned above and to compare the CFU-capacity among the cell lines as well as to identify EPC of high proliferative origin.

UCB-EC, HMVEC and HUVEC were thawed and seeded for up to 48 hours at a density of 1000 to 2000 cells/cm^2 in conventional EGM-2 medium (see appendix). Cells were then trypsinized and seeded at a density of 1 cell/cm^2 in 150 cm^2 culture dishes, again in conventional EGM-2 medium. The medium was changed every three days. After 12 days, cells were washed three times with PBS (pH 7.2 –7.3), fixed with cold Aceton/Methanol (3:2 parts v/v, Merck) for 15 minutes, air dried, rehydrated with deionized water for 10 minutes and stained with Harris` Hematoxylin stain (Merck) for 12 minutes. The numbers of colonies were determined by visual inspection as well as counted using a ChemiDoc™ XRS Universal hood II and analysing software or an AID BacSpot™ System with a colony/CFU being defined as consisting of at least 50 cells. Colonies with a size larger than 2.00 mm in diameter were regarded as being derived from EPC with high proliferative potential (HPP) and their number was also counted (so called HPP CFU-EPC). The number of HPP CFU-EPC was expressed as % of all colonies formed by EPC (CFU-EPC).

Table 1 shows, that all three cell lines contain EPC capable of forming colonies with HUVEC showing the highest overall number of colonies. UCB-EC of later passages contained the highest % of HPP CFU-EPC among the cells tested.

Table 1: result of an experiment performed in triplicates:

Cells	HUVEC	HMVEC	UCB-EC	UCB-EC	UCB-EC
Passage	3	2	3	4	5
CFU-EPC : Mean	97	42	59	17	27
CFU-EPC: Std error of mean	6,81	5,24	8,72	2,19	2,00
HPP CFU-EPC: mean	12	3	6	8	7
HPP CFU-EPC : Std error of mean	0,88	0,58	1,00	1,45	1,73
%HPP / CFU-EPC	12,37	7,14	10,17	44,23	25,93

Example 4: Use of HPL-supplemented medium in comparison to FBS-supplemented medium in the determination of CFU-EPC

It was the purpose of this example to analyze if there was any influence on the colonies formed by EPC if the medium was supplemented with human platelet lysate (HPL) instead of FBS.

Passage 4 UCB-EC were thawed and seeded for about 12 hours at a density of 1000 to 2000 cells/cm^2 either in conventional EGM-2 medium or in HPL EGM-2 medium (see appendix). Cells were then trypsinized and seeded at a density of 1 cell/cm^2 in 150 cm^2 culture dishes, again either in conventional EGM-2 medium or in HPL EGM-2 medium. The corresponding medium was changed every three days. After 12 days, cells were washed three times with PBS (pH 7.2 –7.3), fixed with cold

Aceton/Methanol (3:2 parts v/v, Merck) for 15 minutes, air dried, rehydrated with deionized water for 10 minutes and stained with Harris` Hematoxylin stain (Merck) for 12 minutes. Pictures of stained dishes were taken (figure 3).

The use of HPL instead of FBS as supplement in EGM-2 resulted in a strong increase in the numbers as well as the sizes of colonies. Therefore, in case HPL EGM-2 medium was used, colonies with a size larger than 4.00 mm in diameter instead of 2.00 mm were defined as being derived from EPC with high proliferative potential (HPP CFU-EPC, see example 5).

Example 5: Influence of certain growth factors in HPL-supplemented medium on the quantity and absolute cell numbers of CFU-EPC

It was the purpose of this example to analyze if there was any influence on the colonies formed by EPC if the medium (supplemented with HPL) lacked human epidermal growth factor (EGF), human fibroblast growth factor B (FGF) and the R3-insulin like growth factor 1.

UCB-EC and HUVEC were thawed and seeded for about 24 hours at a density of 1000 to 2000 cells/cm^2 either in HPL EGM-2 medium or HPL-3 EGM-2 medium (see appendix). Cells were then trypsinized and seeded at a density of 1 cell/cm^2 in 150 cm^2 culture dishes, again either in HPL EGM-2 medium or HPL-3 EGM-2 medium. The corresponding medium was changed every 3 days. After 12 days, cells were washed three times with PBS (pH 7.2 –7.3), fixed with cold Aceton/Methanol (3:2 parts v/v, Merck) for 15 minutes, air dried, rehydrated with deionized water for 10 minutes and stained with Harris` Hematoxylin stain (Merck) for 12 minutes. The numbers of colonies as well as the numbers of cells in total were determined by visual inspection as well as counted using a ChemiDoc™ XRS Universal hood II and analysing software or an AID BacSpot™ System with a colony being defined as

consisting of at least 50 cells. Figure 4 shows the results and corresponding pictures of the stained culture dishes.

The quantity of colonies in both cell types was higher using fully supplemented HPL EGM-2 medium. This correlated with a strong increase of the total cell numbers on plates if HPL EGM-2 medium was used (for HUVEC a 467 fold and for UCB-EC a 604 fold increase) compared to an increase 17 fold in HUVEC and 71 fold in UCB-EC if HPL-3 EGM-2 medium was used (figure 4). However, the cells were growing and colonies were detectable in case HPL EGM-2 medium lacking EGF, FGF and R3-insulin like growth factor 1 was used for culturing. Therefore, those three growth factors can, in principle, be omitted from the medium containing HPL as supplement.

Example 6: Comparison of fully supplemented HPL EGM-2 medium, HPL EGM-2 medium lacking certain growth factors and fully supplemented FBS EGM-2 medium for the use in CFU-EPC assays

It was the purpose of this example to compare the numbers and sizes of colonies of HUVEC and UCB-EC in CFU-EPC assays using three EGM-2 media of different compositions.

Passage 4 UCB-EC and passage 4 HUVEC were thawed and seeded for about 12 hours at a density of 1000 to 2000 cells/cm^2 either in conventional EGM-2 medium, HPL EGM-2 medium or HPL-3 EGM-2 medium (see appendix). Cells were then trypsinized and seeded at a density of 1 cell/cm^2 in 150 cm^2 culture dishes, again either in conventional EGM-2 medium, HPL EGM-2 medium or HPL-3 EGM-2 medium. The corresponding medium was changed every three days. After 12 days, cells were washed three times with PBS (pH 7.2 –7.3), fixed with cold Aceton/Methanol (3:2 parts v/v, Merck) for 15 minutes, air dried, rehydrated with deionized water for 10 minutes and stained with Harris` Hematoxylin stain (Merck)

for 12 minutes. The numbers of colonies were determined by visual inspection as well as counted using a ChemiDoc™ XRS Universal hood II and analysing software or an AID BacSpot™ System with a colony being defined as consisting of at least 50 cells. Colonies were also classified according to their size in the following categories as indicated in figure 5: smaller than 2 mm, between 2 mm and 4 mm and larger than 4 mm.

The use of HPL EGM-2 resulted in the largest number of colonies with the biggest sizes. Interestingly, the use of HPL EGM-2 medium lacking EGF, FGF and R3-like insulin growth factor 1 showed results comparable to the use of conventional, fully supplemented FBS EGM-2 medium (figure 5).

Example 7: Analysis of the influence of EPO on the CFU of epithelial progenitor cells

It was the purpose of this example to analyze the influence of EPO on the overall colony number of EPC and the colony number derived from HPP-EPC among the CFU-EPC. In this example, EPO represents a test compound, which can be chosen from a large group of compounds with possible influence on the CFU-EPC. This group comprises small molecules from molecule libraries, growth factors and compounds of pharmaceutical interest.

UCB-EC, HMVEC and HUVEC were thawed and seeded for about 12 hours at a density of 1000 to 2000 cells/cm^2 in conventional EGM-2 medium (see appendix). Cells were then trypsinized and seeded at a density of 1 cell/cm^2 in 150 cm^2 culture dishes, again in conventional EGM-2 medium either without or with EPO (Janssen-Cilag) in different concentrations ranging from 1 to $1x10^{-16}$ I.U./ ml as indicated in figures 6 to 10. The corresponding medium was changed every 3 days. After 12 days, cells were washed three times with PBS (pH 7.2 –7.3), fixed with cold

Aceton/Methanol (3:2 parts v/v, Merck) for 15 minutes, air dried, rehydrated with deionized water for 10 minutes and stained with Harris` Hematoxylin stain (Merck) for 12 minutes. The numbers of colonies were determined by visual inspection as well as counted using a ChemiDoc™ XRS Universal hood II and analysing software or an AID BacSpot™ System with a colony being defined as consisting of at least 50 cells. Colonies with a size larger than 2.00 mm in diameter were regarded as being derived from EPC with high proliferative potential (HPP) and also counted (so called HPP CFU-EPC). The number of HPP CFU-EPC was expressed as percent of total colonies formed by EPC (CFU-EPC).

The results are shown in figures 6-10. The addition of EPO in a concentration of 10^{-4} I.U./ml showed a statistically significant reduction of HPP-CFU-EPC in HUVEC, whereas there was no influence on the CFU and HPP-CFU-EPC in HVMEC and if any, only minor influence on the CFU- and HPP-CFU-EPC in UCB-EC of different passages.

Example 8: Preparation of a cell culture supplement comprising plasma-free platelet lysate and human serum albumin

The donated whole blood (450ml) was anticoagulated by addition of 62ml CPD (26.3 g/l sodium dihydrate, 3.27 g/l citric acid monohydrate, 25.5 g/l glucose monohydrate and 2.51 g/l sodium dihydrogenphosphate dihydrate). After a resting period of 16 hours at 22±2°C the blood units were centrifuged at 4247g at 22°C for 10 minutes. Erythrocytes and plasma were separated automatically (Compomat G3, NPBI, Amsterdam, The Netherlands) from the BC fraction and transferred into satellite containers. Randomised buffy coats from four different ABO- and Rhesus-identical donations and one bag containing plasma from one of the four donors were connected sterilely (TSCD, Terumo Corp., Tokyo, Japan) in series and pooled by gravity in the lowest container. The pooled BCs were centrifuged at 341g at 22°C for

6 minutes and the platelet-rich plasma was leukocyte-depleted by inline filtration (PALL Autostop, Pall, Dreieich, Germany) and transferred into a platelet storage bag (ELX, PALL, Dreieich, Germany). The mean±SD platelet concentration was $0.93\pm0.10x10^9$/ml.

To remove the plasma the pooled concentrate was centrifuged at 2500g for 10 minutes after addition of 10% ACD-A to avoid aggregate formation and the supernatant was discarded. A solution containing 5% human albumin (Immuno Baxter AG, Vienna, Austria) and 10% ACD-A was added to the pellet and the platelet pellet was resuspended to yield a final platelet concentration of 1 - $2x10^9$/ml. The suspension of platelets was frozen at -80°C for lysis of the platelets and release of growth factors. After thawing, several units of platelet lysate were pooled to avoid individual donor variations and the pool was frozen again at -80°C until use.

Appendix: Compositions of media used in the examples

Conventional EGM-2 medium:
(EGM-2 and SingleQuots by Cambrex, FBS by Hyclone)

EGM-2

Hydrocortisone
2% FBS
VEGF
Ascorbic acid
Heparin
Gentamycin
Amphotericin
EGF

FGF
R3-insulin like growth factor 1

10% FBS

Conventional low serum EGM-2 medium:
(EGM-2 and SingleQuots by Cambrex, FBS by Hyclone)

EGM-2

Hydrocortisone
2% FBS
VEGF
Ascorbic acid
Heparin
Gentamycin
Amphotericin
EGF
FGF
R3-insulin like growth factor 1

2% FBS

HPL EGM-2 medium:
(EGM-2 and SingleQuots by Cambrex, HPL prepared according to EP06120857.5):

EGM-2

Hydrocortisone
2% FBS
VEGF
Ascorbic acid
Heparin
Gentamycin
Amphotericin
EGF
FGF
R3-insulin like growth factor 1

7,5% HPL

HPL-3 EGM-2 medium:
(EGM-2 and single stocks by Cambrex, HPL prepared according to EP06120857.5):

EGM-2

Hydrocortisone
2% FBS
VEGF
Ascorbic acid
Heparin
Gentamycin
Amphotericin

7,5% HPL

CLAIMS

1. A method of determining the colony forming units (CFU) of endothelial progenitor cells (EPC), which comprises the following steps:

    a. Seeding cells not modified to express exogenous proteins as markers for identification and sorting purposes in an appropriate medium at such a density, that contact inhibition cannot take place;

    b. Incubating cells for several days; and

    c. Identifying colonies.

2. A method of identifying compounds and/or conditions exerting an effect on the CFU of EPC, which comprises the following steps:

    a. Seeding cells in an appropriate medium at such a density, that contact inhibition cannot take place;

    b. Contacting cells with one or several test compound(s) and/or test condition(s)

    c. Incubating cells for several days;

    d. Identifying colonies; and

    e. Comparing with a control.

3. A method according to claims 1 and 2, wherein the cells comprise HUVEC, HMVEC and endothelial cells prepared from umbilical cord blood (UCB-EC) as well as cells derived from any mammalian solid organ or vasculature, such as bone marrow or lung.

4. A method according to the preceding claims, wherein the appropriate medium comprises EGM-2, hydrocortisone and VEGF and is supplemented with FBS.

5. A method according to claim 4, wherein the appropriate medium is supplemented with HPL instead of FBS.

6. A method according to claims 4 or 5, wherein the appropriate medium additionally comprises EGF, human fibroblast growth factor B and the R3-insulin like growth factor 1.

7. A method according to any of claims 1 to 6, wherein the cells are seeded at a density of 1 cell/cm^2 in a 150 cm^2 culture dish and incubated for 12 days with a medium change every third day.

8. A method according to any of claims 1 to 7, wherein the colonies are identified by staining the cells with Harris` Hematoxylin staining.

9. A method according to any of claims 1 to 8, wherein a colony is defined according to its size as derived from a regular CFU or a CFU with high proliferative potential (HPP).

10. A method according to claim 9, wherein a HPP-CFU is defined as having a colony size > 2.0 mm and a regular CFU is defined as having a colony size <= 2.0 mm if medium according to claim 4, further comprising EGF, human fibroblast growth factor B and the R3-insulin like growth factor I or any medium comprising HPL as supplement but lacking EGF, human fibroblast growth factor B and the R3-insulin like growth factor 1 is used.

11. A method according to claim 9, wherein a HPP-CFU is defined as having a colony size > 4.0 mm and a regular CFU is defined as having a colony size <= 4.0 mm if medium comprising EGM-2, hydrocortisone, VEGF, EGF,

human fibroblast growth factor B, the R3-insulin like growth factor 1 supplemented with HPL is used.

12. A method according to claim 2, wherein a control is defined as an assay under identical conditions, wherein the appropriate medium does not contain one or several test compound(s).

13. A method according to claim 2, wherein a test compound is defined as a positive regulator of the CFU-EPC when increasing the number of CFU compared to a control.

14. A method according to claim 2, wherein a test compound is defined as a negative regulator of the CFU-EPC when decreasing the number of CFU compared to a control.

15. A method according to claim 2, wherein the test compound(s) comprises small molecules derived from small molecule libraries, growth factors and compounds of pharmaceutical interest.

16. Medium comprising EGM-2, hydrocortisone and VEGF, supplemented with HPL.

17. Medium according to claim 16, also comprising ascorbic acid and heparin.

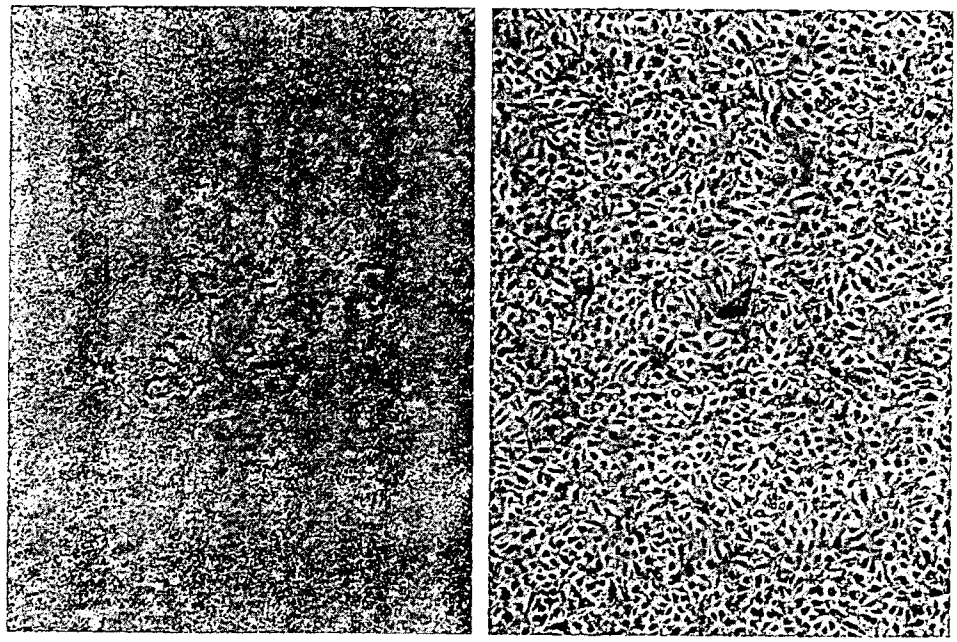
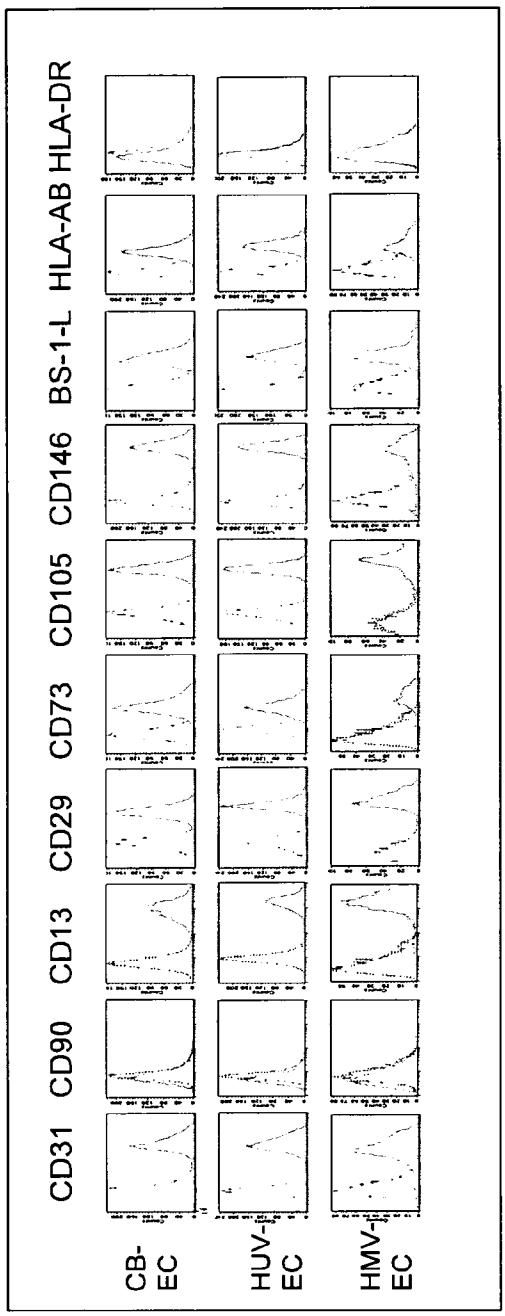


Figure 1

Figure 2


CD31
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CD73
CD105
CD146
BS-1-L
HLA-AB
HLA-DR
CB-EC
HUV-EC
HMV-EC

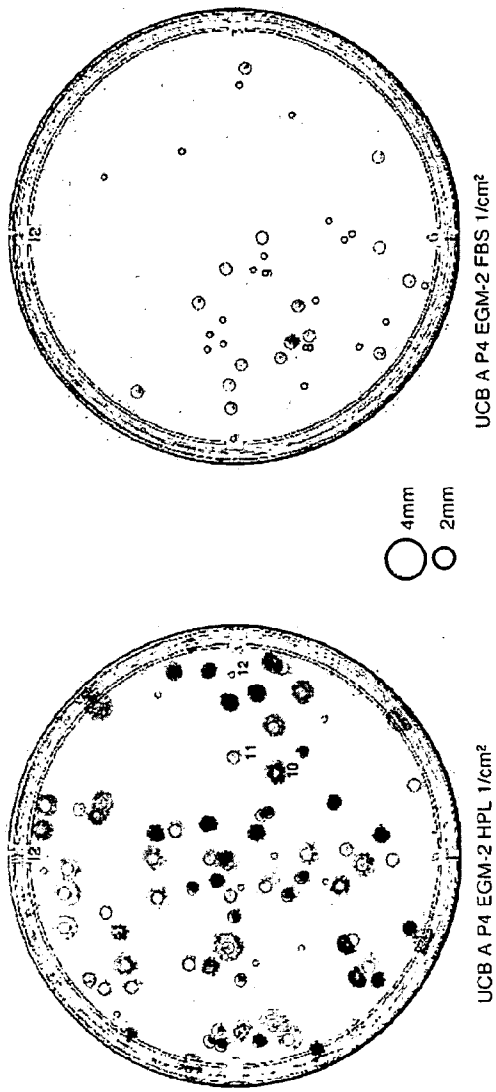

Figure 3

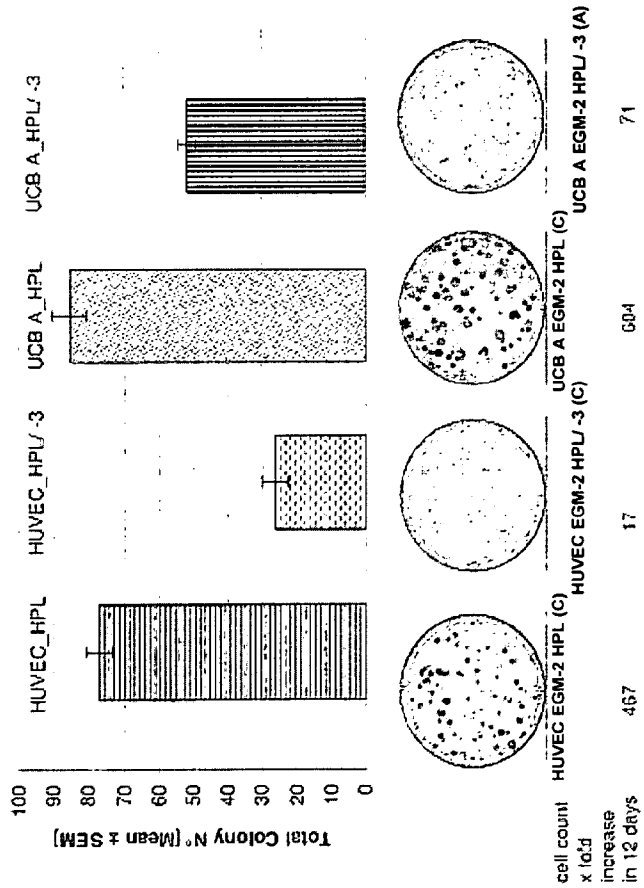
Total Colony N° (Mean ± SEM)
100
90
80
70
60
50
40
30
20
10
0
HUVEC_HPL
HUVEC_HPL/-3
UCB A_HPL
UCB A_HPL/-3
cell count x fold increase in 12 days
467
17
604
71
HUVEC EGM-2 HPL (C)
HUVEC EGM-2 HPL/-3 (C)
UCB A EGM-2 HPL (C)
UCB A EGM-2 HPL/-3 (A)

Figure 4

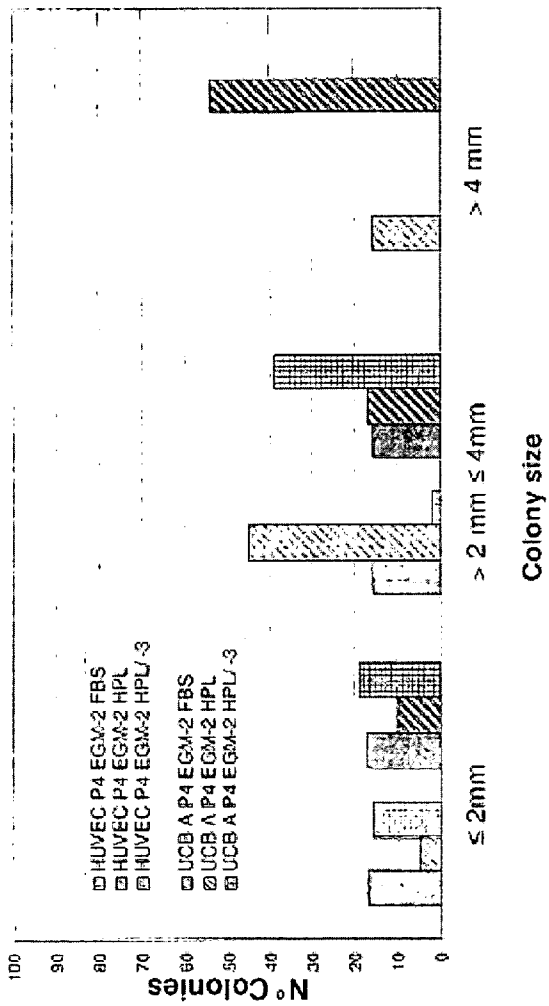
CFU - EPC
N° Colonies
100
90
80
70
60
50
40
30
20
10
0
HUVEC P4 EGM-2 FBS
HUVEC P4 EGM-2 HPL
HUVEC P4 EGM-2 HPL/-3
UCB A P4 EGM-2 FBS
UCB A P4 EGM-2 HPL
UCB A P4 EGM-2 HPL/-3
≤ 2mm
> 2 mm ≤ 4mm
> 4 mm
Colony size

Figure 5

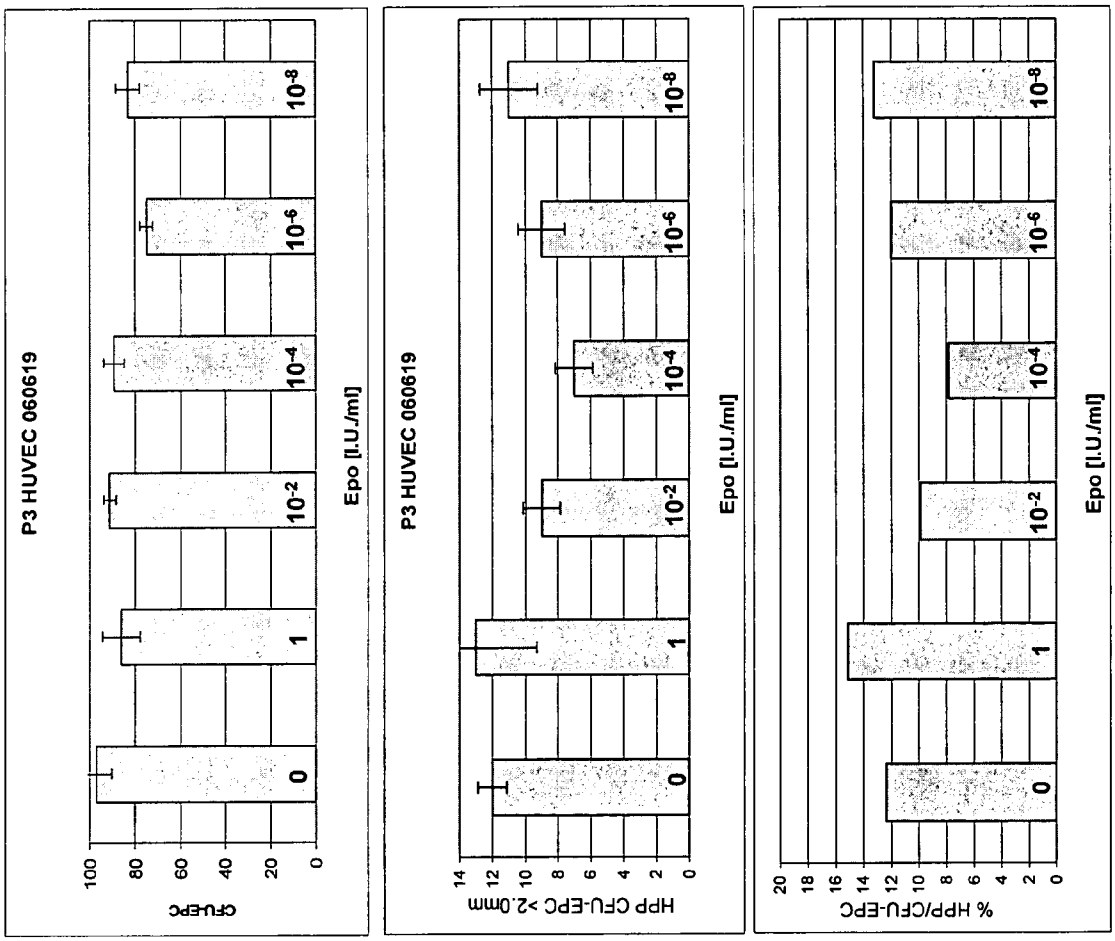
P3 HUVEC 060619
CFU-EPC
Epo [I.U./ml]
0
1
10-2
10-4
10-6
10-8
P3 HUVEC 060619
HPP CFU-EPC >2.0mm
Epo [I.U./ml]
% HPP/CFU-EPC
Epo [I.U./ml]

Figure 6

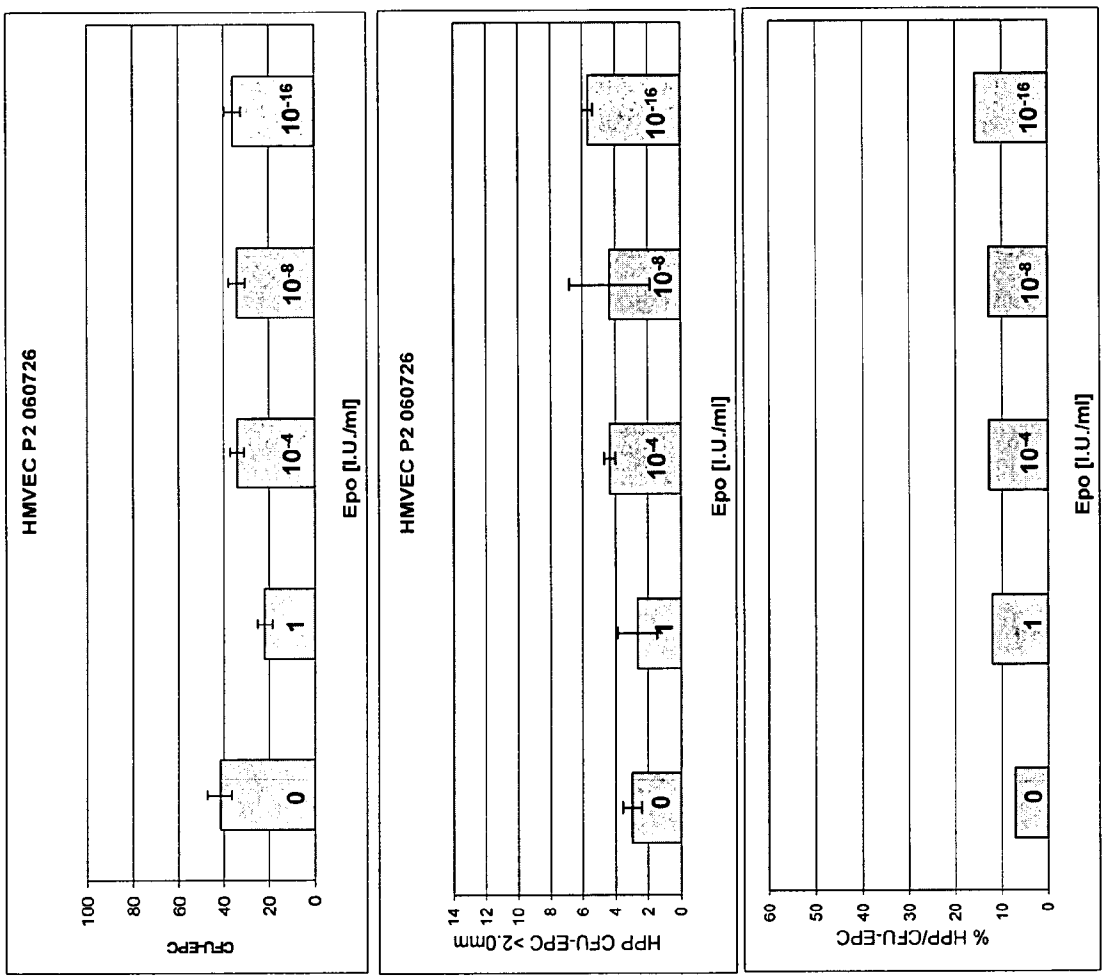
HMVEC P2 060726
CFU-EPC
100
80
60
40
20
0
0
1
10-4
10-8
10-16
Epo [I.U./ml]
HMVEC P2 060726
HPP CFU-EPC >2.0mm
14
12
10
8
6
4
2
0
0
1
10-4
10-8
10-16
Epo [I.U./ml]
% HPP/CFU-EPC
60
50
40
30
20
10
0
0
1
10-4
10-8
10-16
Epo [I.U./ml]

Figure 7

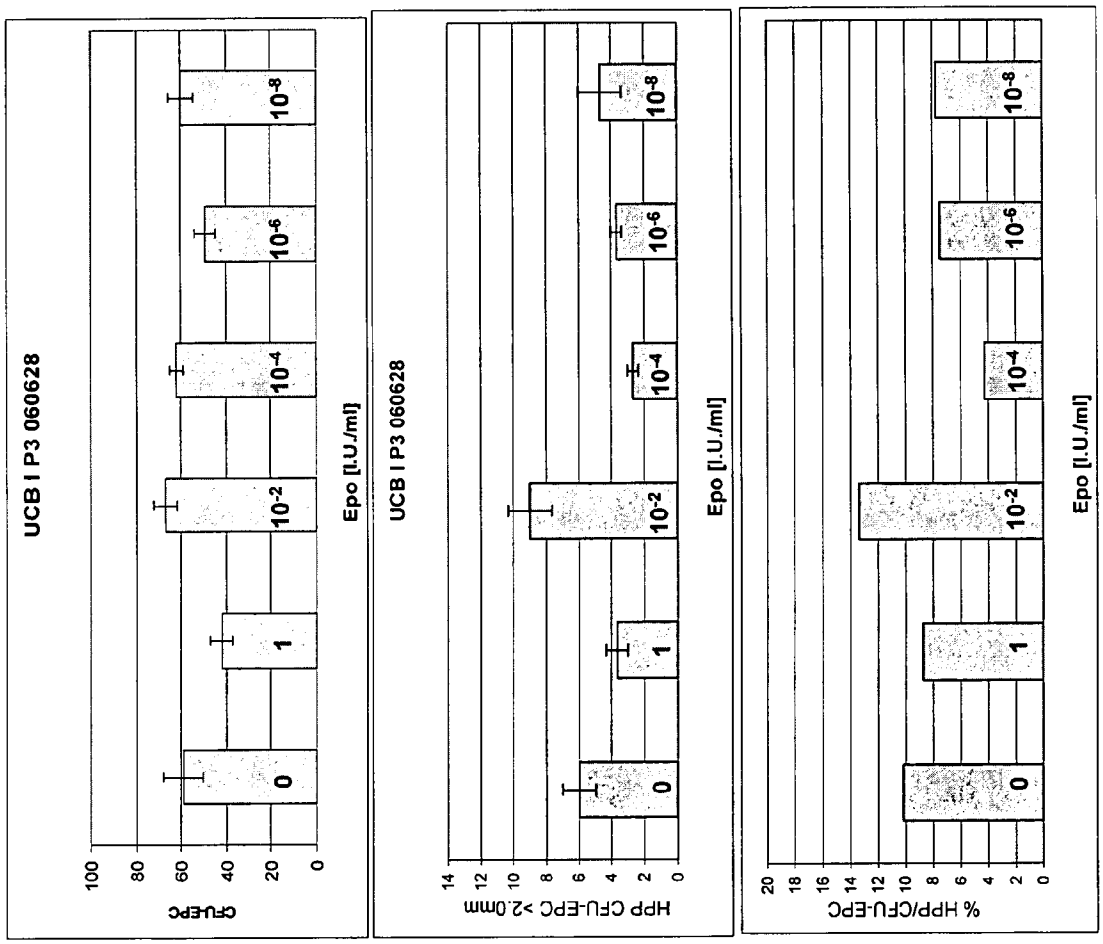
UCB I P3 060628
CFU-EPC
Epo [I.U./ml]
0
1
10-2
10-4
10-6
10-8
UCB I P3 060628
HPP CFU-EPC >2.0mm
Epo [I.U./ml]
% HPP/CFU-EPC
Epo [I.U./ml]

Figure 8

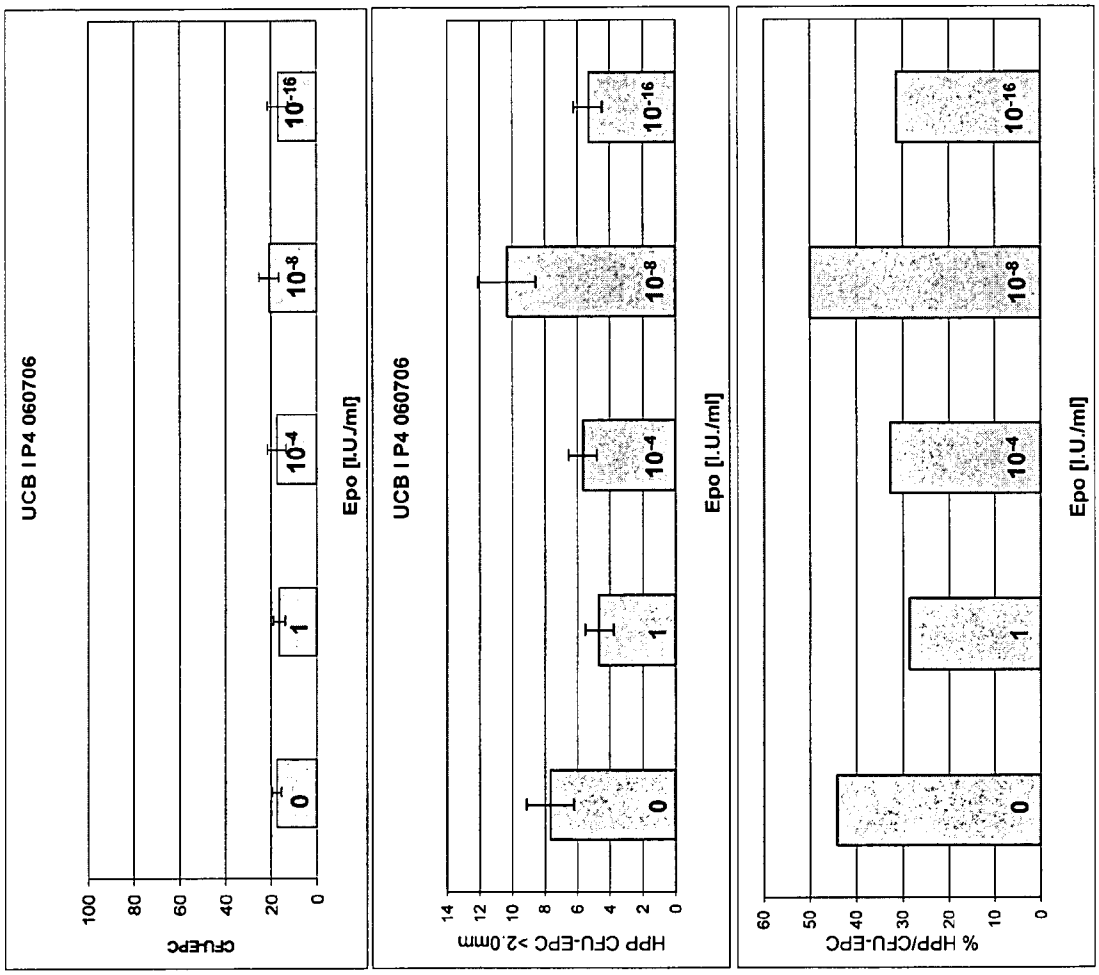
UCB I P4 060706
CFU-EPC
Epo [I.U./ml]
0
1
10-4
10-8
10-16
UCB I P4 060706
HPP CFU-EPC >2.0mm
Epo [I.U./ml]
% HPP/CFU-EPC
Epo [I.U./ml]

Figure 9

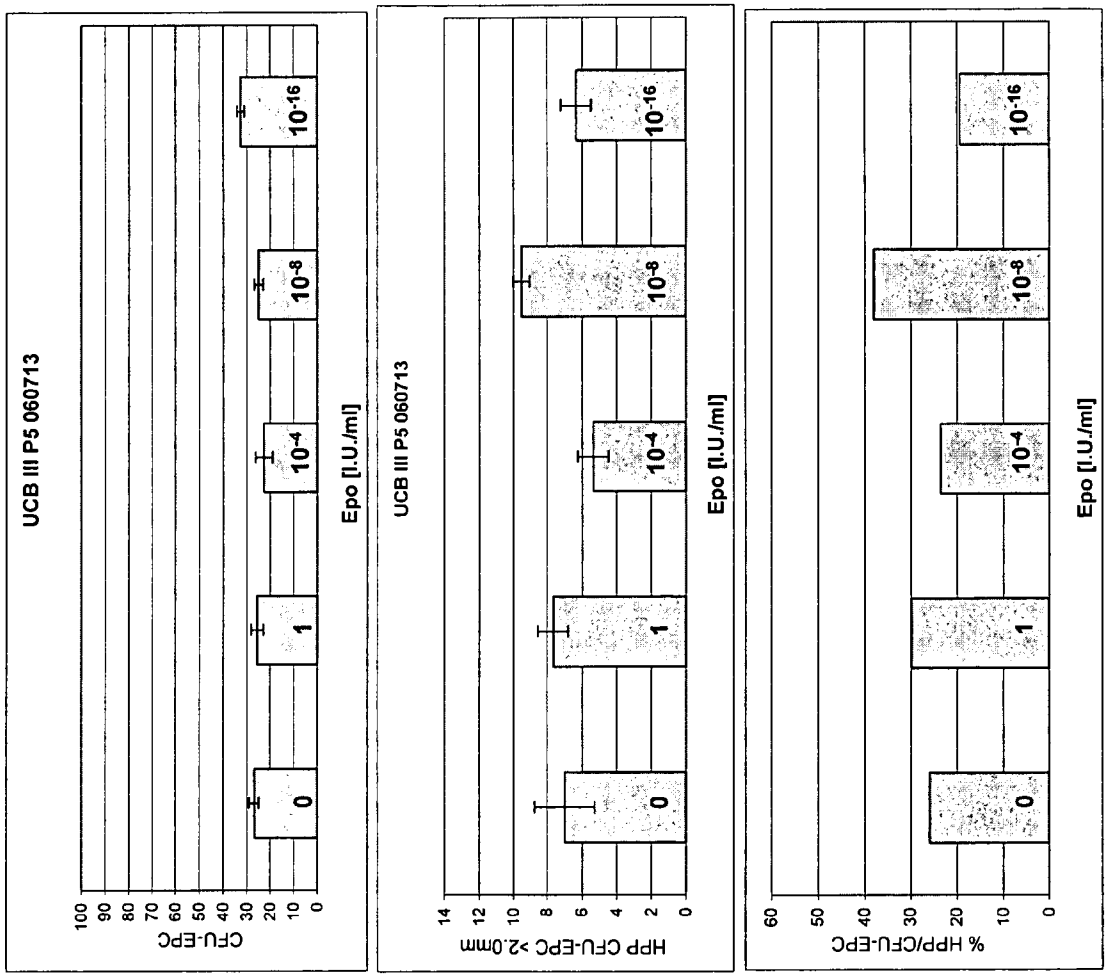
UCB III P5 060713
CFU-EPC
Epo [I.U./ml]
0
1
10-4
10-8
10-16
UCB III P5 060713
HPP CFU-EPC >2.0mm
Epo [I.U./ml]
% HPP/CFU-EPC
Epo [I.U./ml]

Figure 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/052933

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/50 C12N5/06

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IMANISHI ET AL: "Sirolimus accelerates senescence of endothelial progenitor cells through telomerase inactivation" ATHEROSCLEROSIS, AMSTERDAM, NL, vol. 189, no. 2, December 2006 (2006-12), pages 288-296, XP005722975 ISSN: 0021-9150 abstract figure 7 page 289, left-hand column page 290 page 292, left-hand column, paragraph 3 - page 293, left-hand column, paragraph 1 page 293, right-hand column, paragraph 1 page 295, left-hand column, paragraph 2 ----- -/--	1-17

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 April 2008	29/04/2008

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Boiangiu, Clara

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/052933

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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X	HEESCHEN C ET AL: "Erythropoietin is a Potent Physiologic Stimulus for Endothelial Progenitor Cell Mobilization" BLOOD, W.B.SAUNDERS COMPANY, ORLANDO, FL, US, vol. 102, no. 4, 2003, pages 1340-1346, XP002993403 ISSN: 0006-4971 abstract page 1341, left-hand column page 1342, left-hand column, paragraph 1 - page 1343, right-hand column, paragraph 2 figures 1,5	1-17
X	ITO H ET AL: "Endothelial progenitor cells as putative targets for angiostatin" CANCER RESEARCH, AMERICAN ASSOCIATION FOR CANCER RESEARCH, BALTIMORE, MD, US, vol. 59, 1 December 1999 (1999-12-01), pages 5875-5877, XP002954634 ISSN: 0008-5472 the whole document	1-17
X	WO 02/092108 A (CHILDRENS MEDICAL CENTER [US]) 21 November 2002 (2002-11-21) paragraphs [0014], [0020], [0029] - [0036] figures 1,2,4 claim 5	1-17
X	WO 2004/045517 A (US GOVERNMENT [US]; FINKEL TOREN [US]; QUYYUMI ARSHED A [US]; HILL JON) 3 June 2004 (2004-06-03)	1
Y	figures 2,3 page 2, line 25 - page 5, line 25 pages 17-19 page 22, line 13 - page 24, line 14 page 25, line 23 - page 26, line 27 claims 1-53	2-17
X	WO 2005/078073 A (INDIANA UNIVERSITY RES AND TEC [US]; YODER MERVIN C [US]; INGRAM DAVID) 25 August 2005 (2005-08-25)	1
Y	paragraphs [0012] - [0024], [0030] - [0032], [0036] - [0039], [0043] - [0046], [0052] - [0056], [0067] - [0077], [0081] - [0104] claims 13-19	2-17

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2008/052933

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MARCHETTI VALENTINA ET AL: "Benfotiamine counteracts glucose toxicity effects on endothelial progenitor cell differentiation via Akt/FoxO signaling." DIABETES AUG 2006, vol. 55, no. 8, August 2006 (2006-08), pages 2231-2237, XP002476897 ISSN: 0012-1797 abstract page 2231, right-hand column, paragraph 4 - page 2232, left-hand column, paragraph 1 page 2233, right-hand column - page 2234, left-hand column figures 1,3	2-17
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P,X	EP 1 860 196 A (FOUNDATION FOR BIOMEDICAL RES [JP]; TOKAI UNIVERSITY EDUCATIONAL S [JP]) 28 November 2007 (2007-11-28) paragraphs [0006] - [0014], [0036], [0041] - [0061]; claims 1-13; figures 1-11	1-17

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International application No
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