

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
11 December 2008 (11.12.2008)

PCT

(10) International Publication Number
WO 2008/149129 A1

(51) International Patent Classification:
C12N 5/06 (2006.01)

(74) Agents: **DEMPSTER, Robert, Charles** et al.; D Young & Co., 120 Holborn, London EC1N 2DY (GB).

(21) International Application Number:
PCT/GB2008/001988

(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(22) International Filing Date: 9 June 2008 (09.06.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0711111.5 8 June 2007 (08.06.2007) GB
0713299.6 9 July 2007 (09.07.2007) GB

(71) Applicants (*for all designated States except US*): **NOVA THERA LIMITED** [GB/GB]; Minerva Building, Babraham Research Campus, Babraham, Cambridge, CB22 3AT (GB). **IMPERIAL COLLEGE INNOVATIONS LIMITED** [GB/GB]; Electrical and Electronic Engineering Building, South Kensington Campus, London SW7 2AZ (GB).

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **MANTALARIS, Sakis** [GR/GB]; 42 Cornwall Road, Harrow HA1 4NE (GB). **RANDLE, Wesley** [GB/GB]; NovaThera Limited, Minerva Building, Babraham Research Campus, Babraham, Cambridge CB22 3AT (GB).

Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

(54) Title: CELL EXPANSION

(57) Abstract: The present invention provides in one aspect a method of ex-vivo expanding umbilical cord blood CD34+ progenitor cells, comprising: (a) encapsulating umbilical cord blood cells in a support matrix; (b) seeding the encapsulated cells into a dynamic culture vessel; and (c) culturing the cells in the dynamic culture vessel under conditions allowing for CD34+ progenitor cell expansion.



WO 2008/149129 A1

CELL EXPANSION

Field of the invention

The present invention relates to the field of methods for expanding populations of cells, in particular umbilical cord blood stem and/or progenitor cells.

Background of the invention

Cord blood stem cells have been transplanted over 8000 times since 1988 in the treatment of 45 different blood disorders and have been used to repair and restore the bone marrow following high dose chemotherapy for cancer. Nevertheless, the use of cord blood units as a source of stem cells for transplant is restricted because of the relatively low number of CD34+ progenitor cells in a single cord blood unit. An average cord blood unit only contains sufficient CD34+ progenitor cells to treat a patient weighing 30kg. Also, the pooling of cords presents several problems and it is not considered the ideal standard. There is therefore a clear need for CD34+ cell expansion technology to enable cord blood units to be used to treat the full range of patients in need (Kurtzberg *et al.* 1996; Wagner *et al.* 1996; Kapelushnik *et al.* 1998; Shpall *et al.* 2000; Jaroscak *et al.* 2003a).

The *ex vivo* expansion of CD34+ progenitor cells has been attempted in a variety of static and dynamic based systems over recent years, including using bioreactors. These studies have demonstrated the potential of cell expansion but at the same time have not yet led to routine clinical applications.

The continued understanding, discovery and utilisation of cytokines, the isolation and identification of cellular subtypes and in the development of a variety of bioreactor and supporting scaffolds concepts continues to drive the future expansion technologies for CD34+ progenitor cells. Some clinical trials are planned but the technology still needs further refinement (Hoffman *et al.* 1993; Wagner 1993; Andrews *et al.* 1994; Purdy *et al.* 1995; Gehling *et al.* 1997; Bachier *et al.* 1999; Chabannon *et al.* 1999a; McNiece *et al.* 1999; Nielsen 1999; McNiece *et al.* 2000a; McNiece and Briddell 2001; Noll *et al.* 2002; Wolf 2002; Knutsen *et al.* 1998; Vilquin *et al.* 2002).

Lui et al (2006a) have previously attempted to expand cord blood cells encapsulated in calcium alginate in static culture using exogenous cytokines and rabbit bone marrow mesenchymal stem cells. This approach was found to have a negative impact on overall numbers of CD34+ cells and CFU-GM. This is important since expansion with loss of multipotency is of little practical use.

Subsequent work by Lui et al (2006b) used a purpose built rotating wall vessel bioreactor and a cell-dilution feeding protocol with exogenous cytokines but without cell encapsulation.

WO 2005/007799 describes methods for *ex-vivo* expanding stem/progenitor cells in a bioreactor. A wide range of cell types are discussed, such as skin cells, hepatic cells, neural cells and bone cells. Various conditions are employed, including the use of exogenous cytokines and transition metal (notably copper) chelators. Transition metal chelators are used to inhibit differentiation of progenitor cells. However, this document does not specifically teach expanding CD34+ progenitor cells using encapsulation in a dynamic culture vessel.

US6440734 teaches methods and devices for the long-term culture of hematopoietic progenitor cells. This document describes the expansion of cord blood stem cells in a static, tantalum coated scaffold without the use of exogenous cytokines but in the presence of serum. Coating or impregnation of the tantalum coated matrix using a range of biological agents including 'gelatinous agents' is suggested.

WO 2006/081435 teaches a method of providing readily available cellular material derived from cord blood and a composition thereof. This document describes the novel use of electromagnetic radiation in combination with bioreactor technology. No mention is made of encapsulation in this technology.

WO2006/079854 teaches methods for embryonic stem cell culture in which encapsulated embryonic stem cells are maintained and differentiated in 3D culture. However this document provides no teaching relating to cell types such as umbilical cord blood stem or progenitor cells, including CD34+ progenitor cells derived therefrom, since its teaching is restricted to embryonic stem cells.

There are therefore several current methods for the expansion of particular types of stem and progenitor cells. There is nevertheless a clear need for improved methods of growing larger quantities of stem and progenitor cells, particularly CD34+ progenitor cells derived from umbilical cord blood, for clinical transplantation applications and research in regenerative medicine.

Summary of the invention

Accordingly, in one embodiment the present invention provides a method of *ex-vivo* expanding umbilical cord blood CD34+ progenitor cells, comprising: (a) encapsulating umbilical cord blood cells in a support matrix; (b) seeding the encapsulated cells into a dynamic culture vessel; and (c) culturing the cells in the dynamic culture vessel under conditions allowing for CD34+ progenitor cell expansion.

In another embodiment, the present invention provides an encapsulated cell, or expanded population of umbilical cord blood CD34+ progenitor cells obtainable or obtained by a method as defined above.

In a further embodiment, the present invention provides a dynamic culture vessel comprising umbilical cord blood cells encapsulated in a support matrix.

In a further embodiment, the present invention provides an encapsulated cell, or expanded population of umbilical cord blood CD34+ progenitor cells e.g obtainable or obtained by a method as defined above, for use in regenerative therapy.

In a further embodiment, the present invention provides use of an encapsulated cell, or expanded population of umbilical cord blood CD34+ progenitor cells e.g obtainable or obtained by a method as defined above, in regenerative therapy.

In a further embodiment, the present invention provides use of an encapsulated cell, or expanded population of umbilical cord blood CD34+ progenitor cells e.g obtainable or obtained by a method as defined above, for the preparation of a medicament for treating a disease or condition requiring regenerative therapy.

In a further embodiment, the present invention provides a method for treating a subject in need of regenerative therapy, comprising administering a therapeutically

effective amount of progenitor cells obtained by a method as defined above to the subject.

In a further embodiment, the present invention provides a method comprising (a) encapsulating umbilical cord blood cells in a support matrix (e.g. a suitable protective support matrix); and (b) freezing the encapsulated cells.

The cells which are used in the methods of the present invention may be obtained from any species of animal having an umbilical cord, i.e. from any placental mammal. In particular, the cells may be of human, non-human primate, equine, canine, bovine, porcine, caprine, ovine, rodent or murine origin. In one preferred embodiment the cells are obtained from the umbilical cord of an animal from a species which is a member of the family Equidae, more preferably the genus *Equus*, e.g. a horse, zebra or donkey; most preferably the cells are obtained from a horse. In another particularly preferred embodiment the cells are human cells, i.e. are derived from human umbilical cord blood.

Preferably the cells are encapsulated within 72 hours following collection of umbilical cord blood. Since collection of umbilical cord blood typically takes place immediately after delivery, the cells are preferably encapsulated within 72 hours postpartum, more preferably within 48 hours or 24 hours following birth. Alternatively, the umbilical cords may be frozen and the cells collected and encapsulated after thawing.

The support matrix preferably comprises a hydrogel. Preferred materials for use in the support matrix include alginate, fibronectin and methylcellulose, including each of those materials individually or mixtures thereof. The support matrix is preferably in the form of a bead. Hydrogels may present fewer mass transport problems as compared to scaffolds. Also hydrogels can be dissolved allowing for the easy recovery of the cells.

In one embodiment, the cells are encapsulated in a bead, e.g. a hydrogel bead, at a density of 100 to 300,000 cells per bead, e.g. 500 to 300,000, 3000 to 300,000, 3000 to 200,000, 5000 to 200,000, 500 to 100,000, about 5,000 or about 200,000 cells per bead. The mean diameter of the beads may be 0.1 to 5mm, 0.5 to 5 mm, 1 to 5 mm, 2 to 3 mm or about 2.5 mm in particular embodiments.

After encapsulating the cells in the support matrix, the encapsulated cells may, in one embodiment, be frozen. After freezing, the frozen encapsulated cells may be stored for indefinite periods for later use. When required, the frozen encapsulated cells may be thawed before the rest of the method is performed, i.e. before seeding and culturing the thawed encapsulated cells in the dynamic culture vessel. These embodiments enable cord blood expansion to be performed directly from frozen thus limiting post-thaw manipulation and consequent cell loss and damage prior to expansion.

The method is preferably performed in the absence of serum, for example in one embodiment the cells are cultured in a serum free medium. The cells are also preferably cultured in the absence of a transition metal chelator.

In one embodiment the dynamic culture vessel is a bioreactor. More preferably the bioreactor is a rotating wall vessel bioreactor. A particularly preferred dynamic culture vessel for use in the present invention is a HARV bioreactor or a NovaPod™ bioreactor.

The cells may be cultured in a medium which is cytokine-free, or alternatively in a medium comprising one or more cytokines. Where the medium comprises one or more cytokines, in one embodiment the cytokines are early acting cytokines. Examples of suitable early acting cytokines include, but are not limited to, stem cell factor, FLT3 ligand, interleukin-6 and interleukin-3. In another embodiment, the cytokines comprise one or more of stem cell factor (SCF), thrombopoietin (TPO) and granulocyte-colony stimulating factor (G-CSF), for example the medium may comprise SCF, TPO and G-CSF. The cytokine(s) may be present at, for example, a concentration of 1 to 1000, 1 to 500, 1 to 200, 1 to 100, 10 to 100, 1 to 50 or 1 to 10 ng/ml.

In one embodiment, the cells are cultured in the presence of mesenchymal stem cells. The mesenchymal stem cells may either be encapsulated with the cord blood cells or contained within the culture media. The encapsulated cells are preferably cultured in step (c) in a suspension culture.

According to specific embodiments of the present invention, an expanded population of cells (derived from a single umbilical cord blood sample) obtained by performing the present method may be administered to a single individual in need of regenerative

therapy. In other words, progenitor cells found in a cord blood unit taken from a single newborn infant may be expanded sufficiently to provide enough cells to treat a single patient, for example an adult patient. The expanded population of cells may be used to treat any disease or condition requiring regenerative therapy, in particular diseases or conditions requiring treatment with cells of the myeloid lineage. Applications of the expanded cell populations include, but are not limited to, bone marrow transplantation, transfusion medicine, organ repopulation, regenerative medicine, tissue engineering, gene therapy, toxicology and research. Particularly preferred conditions to be treated according to embodiments of the present invention include blood disorders and bone marrow loss following chemotherapy. The present invention can also be used for screening and toxicity studies.

Embodiments of the present invention may thus advantageously enable umbilical cord blood cell populations to be expanded such that they are more useful in therapy. In particular, the use of encapsulation in a support matrix in combination with a dynamic culture vessel may recreate *ex vivo* an environment which is particularly suited to umbilical cord blood CD34+ progenitor cell expansion, whilst inhibiting cell differentiation. Furthermore, the methods of the present invention do not require the use of agents such as serum or transition metal chelators, the presence of which may be problematic for downstream applications such as human cell transplant therapy.

Brief description of the drawings

Aspects of the present invention will now be described, by way of example only, with reference to the following non-limiting specific embodiments and drawings, in which:

Figure 1 shows the results of flow cytometry of CD34+ cell populations before and after cell expansion;

Figure 2 shows morphological images of cellular growth within beads after 10 days of culture of CD34+ cell populations with cytokines;

Figure 3 shows images of live/dead staining of CD34+ cell populations to indicate cell viability at various times after the start of cell culture, in the presence or absence of cytokines;

Figure 4 shows morphological images of 3D cell colonies after cell expansion without refreshing the culture media. Differentiation is observed indicating potency has been maintained.

Detailed description of the invention

The present invention involves methods of *ex-vivo* expansion and culture of progenitor and stem cells from umbilical cord blood and to their possible uses. In particular, in one embodiment human umbilical cord blood CD34+ progenitor cells (as present for example in the buffy coat) can be expanded *ex-vivo* and grown in large numbers, according to the methods of the present invention, in bioreactors. In particular, in one embodiment the method may be used for large scale *ex-vivo* expansion of stem and progenitor cells, resulting in renewable populations of large numbers of stem and/or progenitor cells which may be used in applications such as, for example, bone marrow transplantation, transfusion medicine, organ repopulation, regenerative medicine, tissue engineering, gene therapy, toxicology and research. Significantly, embodiments of the present method are stroma-cell free. Also, embodiments do not require the passaging of cells.

In some embodiments, the method may involve, for example, a 2-100 times, e.g. a 2-50 times, a 2-20 times or a 5 to 20 times expansion of cord blood CD34+ progenitor cells in 2 to 30 days, preferably 5-10 times expansion in about 10 days. In order to provide sufficient cells for transplantation an average cord blood unit of 3×10^8 total nucleated cells must be expanded 4-5 times. Nevertheless, the key parameter is total CD34+ progenitor cells as expansion of total nucleated cells may include very few expanded CD34+ progenitor cells. An average cord blood unit contains 2×10^6 CD34+ progenitor cells (i.e. less than 1% total nucleated cells) sufficient for a patient weighing a maximum of 20kg at a dose rate of 1×10^5 CD34+ cells/kg. A 5-6 times expansion of CD34+ progenitor cells is therefore required to make the cord blood unit clinically useful to the average adult patient. Preferably the expanded cells retain multipotency.

Embodiments of the present invention address the shortcomings of the presently known configurations by providing a method of expanding cord blood CD34+ progenitor cells in a bioreactor.

Definitions

As used herein the term "ex-vivo" refers to a process in which cells are removed from a living organism and are propagated outside the organism.

As used herein, "cell expansion" or "expanding" in relation to the methods of the invention refers to a process of cell proliferation substantially devoid of cell differentiation. Cells that undergo expansion hence maintain their cell renewal properties.

"Progenitor cell" refers herein in general to an immature or undifferentiated cell which may be capable of self renewal and may differentiate into various adult cell types. Some types of hematopoietic progenitor cells may alternatively be referred to as stem cells.

"Umbilical cord blood CD34+ progenitor cells" are cells expressing the CD34 antigen, which are derived from hematopoietic stem cells in umbilical cord blood. They may be capable of giving rise to cells of the myeloid and/or lymphoid lineage.

"Mesenchymal stem cells" are pluripotent blast cells found in the bone marrow, blood, dermis and other locations, which are capable of differentiating into multiple tissue types.

By "encapsulated" or "encapsulating" it is meant that the cell or cells are entirely embedded within the support matrix. The shape of the bead is not particularly relevant, provided that the dimensions, e.g. surface area to volume ratio, are such that nutrients, metabolites, cytokines etc., can readily diffuse into/out of the bead to reach the cell or cells embedded within the bead.

"Dynamic culture vessel" is used herein to refer to any type of vessel suitable for culturing cells under non-static conditions. A dynamic culture vessel typically maintains a homogeneous environment for cell growth by means of a stirring or mixing mechanism, and variables such as temperature and pH may be monitored and

controllable. A dynamic culture vessel typically comprises one or more active means for maintaining stable environmental conditions within the vessel (e.g. it does not rely on diffusion for transport of nutrients within the vessel), and may allow continuous feeding of cultured cells.

As used herein "bioreactor" refers to a device in which biological and/or biochemical processes develop under monitored and controlled environmental and operating conditions, for example pH, temperature, pressure, nutrient supply and waste removal.

Hematopoietic stem cells (HSC) are stem cells and early precursor cells which give rise to all the blood cell types that include both the myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets and some dendritic cells) and lymphoid lineages (T-cells, B-cells, NK-cells, some dendritic cells). As stem cells, they are defined by their ability to form multiple cell types (multipotency) and their ability to self-renew. Individual HSC have the ability to give rise to any of the end-stage blood cell types. During differentiation, daughter cells derived from HSC undertake a series of commitment decisions, retaining differentiation potential for some lineages while losing others. Intermediate cells become progressively more restricted in their lineage potential, until eventually lineage-committed end stage cells are generated.

In humans, white blood cells (hematopoietic mononuclear cells) comprise a mixture of hematopoietic lineages. Over 99% of mononuclear cells are lineage committed and differentiated cells. Subtypes of hematopoietic stem and progenitor cells, and their lineage potential, may be identified by the presence of various antigenic markers on their surface. CD34 is a cell surface glycoprotein which may mediate cell adhesion, and which is typically used as a marker for undifferentiated hematopoietic stem and progenitor cells. CD133 is another glycoprotein found on hematopoietic stem cells which may be used as a marker of undifferentiated cells. CD38 is a glycoprotein found on the surface of many mononuclear cells, which is used as a marker of differentiated cells. Non-lineage committed and/or undifferentiated stem and progenitor cells also typically express low levels of markers such as thy-1 and CD71, which designate a more mature progenitor population. Various hematopoietic cell types may be identified by other markers, such as CD13 & CD33 for myeloid, CD71

for erythroid, CD19 for B cells, CD61 for megakaryocytes, Mac-1 (CD11b/CD18) for monocytes, Gr-1 for granulocytes, or CD3, CD4, CD5 and CD8 for T cells.

According to the present invention, the CD34+ progenitor cells are preferably non-lineage committed progenitor cells, e.g. CD34+ lin-, CD34+CD38- or CD34+CD133+ comprising less than 1% of total mononuclear cells. However, in alternative embodiments the invention may also be used to expand lineage committed progenitor cells, for instance CD34+CD33+ (myeloid committed progenitor cells), CD34+CD3+ (lymphoid committed progenitor cells) and CD34+CD61+ (megakaryocytic committed cells).

The CD34+ progenitor cells used in the methods of the present invention are derived from umbilical cord blood cells. In some embodiments, umbilical cord blood samples may be subjected to a selection, purification or sorting procedure to provide a sample enriched in CD34+ progenitor cells. The cell sample enriched in CD34+ progenitor cells may then be used in the methods of the invention, for instance for as the umbilical cord blood cells used in the encapsulation step.

Methods for purifying or extracting hematopoietic stem and/or progenitor cells from umbilical cord blood are known in the art. For instance, hematopoietic mononuclear cells may be obtained from a blood sample by applying the blood sample onto a Ficoll-Hypaque layer and collecting, following density-cushion centrifugation, the interface layer present between the Ficoll-Hypaque and the blood serum. The interface layer essentially consists of white blood cells present in the blood sample. "Buffy coat cells" is used herein to refer to the population of mostly leukocytes which may be found in the buff-coloured fraction of a centrifuged blood sample, present between the clear and red layers. For the avoidance of doubt, the cord blood buffy coat cells which may be used in the methods of the invention may be obtained by any processing protocol. In other words, "buffy coat cells" refers to cells of the type found in the buff-coloured fraction of a centrifuged blood sample, without requiring that the cells are actually obtained by such a method.

Hematopoietic mononuclear cells, e.g. buffy coat cells, derived from umbilical cord blood samples, may be used as the umbilical cord blood cells referred to in step (a) in preferred embodiments of the methods of the present invention, since they comprise

CD34+ progenitor cells. Alternatively, CD34+ progenitor cells may be further enriched by, for example, differential density centrifugation and/or immunoseparation, e.g. using immunomagnetic separation or flow cytometry/fluorescence-activated cell-sorting (FACS). Antibodies which are selective for hematopoietic stem and progenitor cell types, e.g. monoclonal anti-CD34 and/or anti-CD38 antibodies, may be used in the selection/enrichment procedure. Such antibodies are commercially available and/or may be obtained by standard techniques.

The medium used for growth of the cells to increase numbers of cells within the support matrix structure (i.e. expansion, in which the cells undergo self-renewal by cell division) may be media that supports cell growth, ideally with minimal or no cell differentiation. Various appropriate maintenance media are known in the art for hematopoietic stem cell expansion, such as Stemline II (Sigma, St Louis, MO). The support matrix that encapsulates the umbilical cord CD34+ progenitor cells used in methods according to the present invention, in combination with the use of a dynamic culture vessel, may together inhibit cell differentiation enabling various media to be used. For example, it may not be necessary to include additional specific agents in the culture medium which suppress differentiation, such as cytokines. Suitable environmental conditions for culturing progenitor cells to allow cell expansion are known in the art, for example this step may be performed at 37°C and 5% CO₂. In order to allow expansion of CD34+ cells, the pH in the culture vessel is preferably at least 6.7, more preferably 6.8 to 7.4. Osmolarity is preferably 0.25 to 0.35 mOsmol/kg, more preferably 0.28 to 0.32 mOsmol/kg.

In some embodiments, cytokines may also be included in the culture medium. In one embodiment, one or more cytokines which inhibits or protects against apoptosis (committed cell death) of the cells is used. In one example, the cytokine may be an early acting cytokine.

In further embodiments, the combination and/or concentration of cytokines in the culture medium may differ from the combination of cytokines in the beads, e.g. in the hydrogel.

In one example, early acting cytokines such as, but not limited to, stem cell factor (SCF), FLT3 ligand, interleukin-6 and interleukin-3 may be used. Thus in specific

embodiments the culture medium may comprise one or more of the following concentrations of cytokines: SCF at 50 to 150 ng/ml, Flt3 at 50 to 150 ng/ml, IL-3 at 10 to 100 ng/ml and IL-6 at 10 to 100 ng/ml. Further cytokines may be present in addition to, or instead of, one or more of the above cytokines. For instance, the cytokines may comprise one or more of stem cell factor, thrombopoietin and granulocyte-colony stimulating factor, each in the concentration range of 1 to 1000 ng/ml, e.g. 1 to 500 ng/ml, 10 to 100 ng/ml, 1 to 50 ng/ml or 1 to 10 ng/ml.

Although the use of cytokines is preferred in particular embodiments, the use of too high a concentration is less preferred. A high concentration of growth factors increases the cost of performing the method. Also, in some embodiments too high a concentration could potentially bias cell expansion and differentiation to abnormal routes. Advantageously in specific embodiments of the present invention, a lower concentration of cytokines (than employed in certain methods of the prior art) may be used. For example, in specific embodiments the concentration of each cytokine may be 200 ng/ml or lower, 100 ng/ml or lower, 50 ng/ml or lower, 20 ng/ml or lower, or 10 ng/ml or lower, e.g. 1 to 100 ng/ml, 1 to 50 ng/ml or 1 to 10 ng/ml.

Static culture vessels such as a tissue culture plate allow cells to grow only in 2 dimensions and may suffer from limitations such as the lack of mixing, poor control options and the need for frequent feeding. Processes using bioreactors which provide a dynamic cultivation system, with controlled culture conditions, may enable the expansion of cells in a 3-D environment closer to those in living organisms.

In some embodiments of the present invention, the cells are seeded into and cultured in a stirred flask or spinner flask bioreactor. Stirred bioreactors provide a homogeneous environment and are easy to operate, allowing sampling, monitoring and control of culture conditions. Typical operating modes include batch, fed-batch and perfusion mode (medium exchange with retention of cells by means of an external filtration module or of internal devices such as spin filters).

Spinner flasks are either plastic or glass bottles with a central magnetic stirrer shaft and side arms for the addition and removal of cells and medium, and gassing with CO₂ enriched air. Inoculated spinner flasks are placed on a stirrer and incubated under the appropriate culture conditions. For example, cultures may be stirred at 10-

250, preferably 30-100, and most preferably 50 revolutions per minute. Spinner and stirrer flask systems designed to handle culture volumes of 1-12 liters are commercially available, such as the Coming ProCulture System (Coming, Inc., Acton, MN). Such spinner flasks are typically equipped with probes for monitoring pH, temperatures, oxygen and CO₂ saturation, levels of metabolites such as glucose, nitrogen, amino acids, etc. in the medium, and are in fluid communication, optionally with the aid of a peristaltic pump, with fresh supplies of medium, gases, specific nutrients, and the like, and with waste removal, so that medium can be drawn off or replenished to maintain optimal conditions for stem cell expansion, at a predetermined rate.

Shear stress and turbulent eddies are sometimes a concern with stirred flask bioreactors. Appropriate suspension culture conditions for performing cell culture methods of the invention can be achieved using a low shear, high mixing, dynamic environment. This enables sufficient nutrients and gases to permeate the support matrix structure employed. The dynamic laminar flow generated by a rotating fluid environment is an efficient method for reducing diffusional limitations of nutrients and wastes while minimizing levels of shear. Rotating wall vessels have been used for cell growth in-vitro with a variety of cell types (see, for example, Vunjak-Novalic et al, J Orthop Res 1999;17:130-38, Rhee, et al, In Vitro Cell Dev 2001; 37:127-40, Licato et al In Vitro Cell Dev, 2001;37:121-26 and Pei, et al, FASEB J 2002;16:1691-94). Thus, according to preferred embodiments of the present invention, the bioreactor is a rotating wall vessel bioreactor. Suitable rotating wall vessel bioreactors are well known in the art, for example the HARV (e.g. NASA HARV), RWV Bioreactor, Roller Cell and RCCS-1 from Synthecon (Synthecon Inc, Houston TX), European Space Agency bioreactor (Fokker, Netherlands), or other simulated microgravity or perfused systems, such as airlift bioreactors, and roller bottles of various types from Coming (Coming, Inc., Acton, MN). In a preferred embodiment, the RWV is a HARV from Synthecon (Synthecon Inc, Houston TX). In another embodiment, the bioreactor is a NovaPodTM bioreactor available from NovaThera Limited, Cambridge UK, for instance as described at <http://www.novathera.com>, e.g. http://www.novathera.com/documents/NovaPod_I_Brochure_Medica_07.pdf. Although stirred bioreactors may create shear stress forces, the NovaPod has reduced shear stress. Shear stress is also eliminated by

the encapsulation which offers added protection to the cells without reduced mass transport.

The support matrix utilised for encapsulation is permeable to allow diffusion and mass transfer of nutrients, metabolites, and growth factors. A cell or cells encapsulated within a support matrix can be provided in the form of a bead, e.g. a generally spherical bead. One or a plurality of progenitor cells may be encapsulated into each support matrix structure, such as a bead. Typically each bead may comprise 100 to 1 million viable cells, 3000 to 300,000 cells, 3000 to 200,000 cells, 5000 to 200,000 cells, 5,000 to 500,000 cells, 10,000 to 300,000 cells, 20,000 to 200,000 cells, about 5,000 or about 200,000 cells per bead.

In 2D conventional culture, actual cell density can be $10000 \text{ cells}/0.0018\text{cm}^3$ assuming the cell size is $15 \mu\text{m}$ (0.0015 cm) and the well plate size to be 1.2 cm^2 . In comparison, if 5000 cells are used per bead the actual cell density in 3D culture is $5000\text{cells}/0.036\text{cm}^3$, assuming the diameter of beads to be 2.5 mm . This is approximately a 40 times lower cell density in this 3D culture system when compared to 2D. In some embodiments of the present invention, the use of 5000/cells per bead (2.5mm diameter) allows suitable cell expansion. Higher densities such as 20,000 or 200,000 cells per bead may be used to increase expansion rates by 15-fold to 30-fold respectively. Alternatively, in some embodiments expansion at lower densities may be preferred in view of the low numbers of available cells from umbilical cord samples.

It is particularly preferred that the support matrix structures, e.g. beads, are constructed of a suitable support matrix material that remains intact during the culture time. The cell or cells encapsulated within the support matrix can be placed into a culture vessel such as a RWV bioreactor (Synthecon, USA) or other simulated microgravity or perfused bioreactor and incubated in maintenance and/or differentiation medium without significant damage for prolonged periods.

Preferably the support matrix material consists of or comprises a gel-forming polysaccharide, such as an agarose or alginate, (typically in the range of from about 0.5 to about 2% w/v, preferably at from about 0.8 to about 1.5% w/v, more preferably about 0.9 to 1.2% w/v). The matrix may consist of alginate alone or may comprise

further constituents such gelatin (typically at from about 0.05 to about 2% v/v, e.g. 0.05 to about 1% v/v, about 0.08 to about 0.5% v/v, or about 1% v/v). The inclusion of gelatin assists in production of a uniform bead size and helps to maintain structural integrity. Inclusion of gelatin in alginate support matrix beads enables cell-mediated contraction and packing of the scaffold material.

In preferred embodiments, the support matrix material comprises a) alginate and methylcellulose, or b) alginate and fibronectin, or c) alginate, methylcellulose and fibronectin. The amount of each of these components may be varied in order to achieve a desired consistency and hardness of the support matrix, which is most conducive to cell expansion. Preferably the support matrix comprises 0.01 to 1% v/v methylcellulose, more preferably 0.1 to 1.0% v/v methylcellulose, and most preferably about 0.1% v/v methylcellulose. Preferably the support matrix comprises 0.5 to 500 µg/ml fibronectin, more preferably 1 to 100 µg/ml fibronectin, more preferably 1 to 50 µg/ml fibronectin and most preferably about 50 µg/ml fibronectin. In a particularly preferred embodiment, the support matrix comprises 0.05 to 0.5% v/v methylcellulose and 1 to 100 µg/ml fibronectin.

Alginate is a water-soluble linear polysaccharide extracted from brown seaweed and is composed of alternating blocks of 1-4 linked α -L-glucuronic and β -D-mannuronic acid residues. Alginate forms gels with most di- and multivalent cations, although Ca^{2+} is most widely used.

When the support matrix is in the form of beads comprising a single cell, the beads may be, for example, from about 20 to 150 microns, preferably from about 40 to about 100 microns in diameter. Beads containing a plurality of cells may have a mean diameter of for example, 0.1 to 10mm, 0.5 to 10 mm, 1 to 10 mm, 1 to 5 mm, about 2.0 to about 2.5 millimetres, about 2.3 millimetres or about 2.5 mm.

In some aspects of the invention, it is preferred that the support matrix employed can be readily dissolved to release cells, without the use of trypsinisation. In instances where it is desirable to remove the support matrix to liberate cells, hydrogel matrices, for example alginate and alginate-based matrices, are favoured as they can be readily dissolved using sodium citrate and sodium chloride solutions.

A method or use according to the invention may further comprise freezing the encapsulated cells for storage, for example after the cell culture and expansion step (c), in addition to the optional freezing step after encapsulation step (a). Encapsulated cells can be frozen using standard protocols, and may be frozen in the maintenance or differentiation medium in which they were cultured. A suitable method for freezing encapsulated cells involves cryopreservation in dimethyl sulfoxide (DMSO) using a slow freezing procedure as described by Stensvaag et al (2004) *Cell Transplantation* 13 (1): 35.

Methods of the invention may further comprise liberation of a cell or cells from the support matrix. The present invention therefore provides a cell or cells so obtained. Where alginate or alginate based matrices are used for encapsulation, liberation of cells can be achieved by alginate dissolution. Such gentle dissolution methods may be advantageous compared to standard enzymatic methods, such as trypsinisation, which may affect the behaviour of the cells in long-term cultures.

In some embodiments, the method of the invention may comprise a further step of inducing differentiation of the expanded cell population. Differentiation may be induced while the cells are still encapsulated, or alternatively after liberating the cells from the support matrix.

Removal of encapsulation may, in some embodiments, favour differentiation of the cells. Some cell lines undergo spontaneous differentiation after cycles of cell division in maintenance growth, particularly if the conditions are such that differentiation is not suppressed. Any medium which supports differentiation is suitable for use in the methods of the present invention. The differentiation medium may be similar to that used in the expansion step, except that in embodiments where a substance which suppresses differentiation is included in the medium used for culturing/expanding the progenitor cells, this substance is not included in the differentiation medium. Conditions suitable for cell differentiation may comprise a stimulus for differentiation of the CD34+ progenitor cell. The stimulus for differentiation can be a stimulus for differentiation to, for example, a myeloid or lymphoid hematopoietic lineage. Environmental conditions in the differentiation step may otherwise be similar to those used in the expansion step.

Thus the differentiation and expansion steps may, for example, be performed in the same vessel. Integrated methods of methods of expansion and differentiation are suitably performed in suspension culture in a flask or bioreactor. In the expansion phase the encapsulated progenitor cells divide and cell numbers are increased, so that colonies of cells form within the support matrix structure. The encapsulated cells are then differentiated forming further differentiated or terminally differentiated cells, all within the 3-D matrix structure. In methods of the invention the further differentiated or terminally differentiated cells can then be maintained, allowing the cells to divide so that cell numbers are increased and colonies of cells form within the support matrix structure.

In certain embodiments, an agent that induces differentiation can be added to a population of cells within a container, including, but not limited to, Ca^{2+} , EGF, alpha-FGF, beta-FGF, PDGF, keratinocyte growth factor (KGF), TGF-beta, cytokines (e.g., IL-1alpha, IL-1beta, IFN-gamma, TFN), retinoic acid, transferrin, hormones (e.g., androgen, estrogen, insulin, prolactin, triiodothyronine, hydrocortisone, dexamethasone), sodium butyrate, TPA, DMSO, NMF, DMF, matrix elements (e.g., collagen, laminin, heparan sulfate, or combinations thereof).

The duration of the expansion and differentiation steps is not particularly limited. For example, the expansion step may last at least 1, 5, 10, 30 or 100 days, e.g. 1 to 30 days.

Cultured cells and expanded populations of cells prepared according to the methods of the invention, or differentiated cells obtained therefrom, may be used as a medicament, for instance in regenerative therapy. Typically the cells are administered to a patient following removal of the support matrix, i.e. after releasing the cells from the encapsulation. In general, regenerative therapy includes a wide variety of therapeutic protocols in which a tissue or organ of the body is augmented, repaired or replaced by the engraftment, transplantation or infusion of a desired cell population, such as a stem cell or progenitor cell population. In a preferred embodiment of the invention, the cells are used to treat blood disorders or to repair bone marrow following high dose chemotherapy. Expanded cell populations according to the present invention may be used as autologous and allogenic, including matched and mismatched HLA type hematopoietic transplants.

The cultured cells can be used to repair damage of tissues and organs resulting from disease. In such an embodiment, a patient can be administered cultured progenitor cells to regenerate or restore tissues or organs which have been damaged as a consequence of disease, e.g., to enhance the immune system following chemotherapy or radiation, or to repair heart tissue following myocardial infarction. The cultured cells can be used to augment or replace bone marrow cells in bone marrow transplantation. Human autologous and allogenic bone marrow transplantation are currently used as therapies for diseases such as leukemia, lymphoma and other life-threatening disorders. The drawback of these procedures, however, is that a large amount of donor bone marrow must be removed to insure that there is enough cells for engraftment. The expanded cell populations according to the present invention can provide stem cells and progenitor cells that reduce the need for large bone marrow donation.

Thus in one embodiment, the expanded cell populations can be used in a supplemental treatment in addition to chemotherapy. Most chemotherapy agents used to target and destroy cancer cells act by killing all proliferating cells, i.e., cells going through cell division. Since bone marrow is one of the most actively proliferating tissues in the body, hematopoietic stem cells are frequently damaged or destroyed by chemotherapy agents and in consequence, blood cell production is diminished or ceases. Chemotherapy must be terminated at intervals to allow the patient's hematopoietic system to replenish the blood cell supply before resuming chemotherapy. It may take a month or more for the formerly quiescent stem cells to proliferate and increase the white blood cell count to acceptable levels so that chemotherapy may resume (when again, the bone marrow stem cells are destroyed).

While the blood cells regenerate between chemotherapy treatments, however, the cancer has time to grow and possibly become more resistant to the chemotherapy drugs due to natural selection. Therefore, the longer chemotherapy is given and the shorter the duration between treatments, the greater the odds of successfully killing the cancer. To shorten the time between chemotherapy treatments, expanded progenitor cell populations produced according to the present invention may be introduced into the patient. Such treatment would reduce the time the patient would

exhibit a low blood cell count, and would therefore permit earlier resumption of the chemotherapy treatment.

The present invention also encompasses pharmaceutical compositions comprising expanded populations of CD34+ progenitor cells produced according to the present methods. These cells can be used with, or as a mixture with, other stem cells, for use in transplantation and other uses. The expanded cell populations, typically after being released from the support matrix, may be used unfrozen, or frozen for later use. If the population of cells is to be frozen, a standard cryopreservative (e.g., DMSO, glycerol, Epilife (Registered Trademark) Cell Freezing Medium (Cascade Biologics)) is added to the enriched population of cells before it is frozen.

In certain embodiments, one or more populations of progenitor cells are delivered to a patient in need thereof. In one embodiment, the invention provides a method of treating or preventing a disease or disorder in a subject comprising administering to a subject in which such treatment or prevention is desired a therapeutically effective amount of the progenitor cells obtained by the methods defined herein.

In another embodiment, the invention provides a method of treating or preventing a disease or disorder in a subject comprising administering to a subject in which such treatment or prevention is desired a therapeutically effective amount of CD34+ progenitor cells obtained by the methods of the present invention.

In another embodiment, the expanded cell populations may be used to treat any disease, condition or disorder resulting from, or associated with, inflammation. The inflammation may be present in any organ or tissue, for example, muscle; nervous system, including the brain, spinal cord and peripheral nervous system; vascular tissues, including cardiac tissue; pancreas; intestine or other organs of the digestive tract; lung; kidney; liver; reproductive organs; endothelial tissue, or endodermal tissue.

The cell populations may also be used to treat immune-related disorders, particularly autoimmune disorders, including those associated with inflammation. Thus, in certain embodiments, the invention provides a method of treating an individual having an autoimmune disease or condition, comprising administering to such individual a therapeutically effective amount of progenitor cells obtained by the present methods,

wherein said disease or disorder can be, but is not limited to, diabetes, amyotrophic lateral sclerosis, myasthenia gravis, diabetic neuropathy or lupus, acute or chronic allergies, e.g., seasonal allergies, food allergies, allergies to self-antigens. Other diseases which may be treated include aplastic anemia, myelodysplasia, myocardial infarction, seizure disorder, multiple sclerosis, stroke, hypotension, cardiac arrest, ischemia, inflammation, age-related loss of cognitive function, radiation damage, cerebral palsy, neurodegenerative disease, Alzheimer's disease, Parkinson's disease, Leigh disease, AIDS dementia, memory loss, amyotrophic lateral sclerosis (ALS), ischemic renal disease, brain or spinal cord trauma, heart-lung bypass, glaucoma, retinal ischemia, retinal trauma, lysosomal storage diseases, such as Tay-Sachs, Niemann-Pick, Fabry's, Gaucher's, Hunter's, and Hurler's syndromes, as well as other gangliosidoses, mucopolysaccharidoses, glycogenoses, inborn errors of metabolism, adrenoleukodystrophy, cystic fibrosis, glycogen storage disease, hypothyroidism, sickle cell anemia, Pearson syndrome, Pompe's disease, phenylketonuria (PKU), porphyrias, maple syrup urine disease, homocystinuria, mucopolysaccharidenosis, chronic granulomatous disease and tyrosinemia, Tay-Sachs disease, cancer, tumors or other pathological or neoplastic conditions.

In other embodiments, the cells may be used in the treatment of any kind of injury due to trauma, particularly trauma involving inflammation. Examples of such trauma-related conditions include central nervous system (CNS) injuries, including injuries to the brain, spinal cord, or tissue surrounding the CNS injuries to the peripheral nervous system (PNS); or injuries to any other part of the body. Such trauma may be caused by accident, or may be a normal or abnormal outcome of a medical procedure such as surgery or angioplasty. Trauma may also be the result of the rupture, failure or occlusion of a blood vessel, such as in a stroke or phlebitis. In specific embodiments, the cells may be used in autologous or heterologous tissue regeneration or replacement therapies or protocols, including, but not limited to treatment of corneal epithelial defects, cartilage repair, facial dermabrasion, mucosal membranes, tympanic membranes, intestinal linings, neurological structures (e.g., retina, auditory neurons in basilar membrane, olfactory neurons in olfactory epithelium), burn and wound repair for traumatic injuries of the skin, or for reconstruction of other damaged or diseased organs or tissues.

The dose of the cells according to the present invention which is administered to a subject will depend on the size of the subject and the nature of the condition requiring treatment. Typically, a patient receiving a stem cell infusion, for example for a bone marrow transplantation, receives one unit of cells, where a unit is approximately 10^9 nucleated cells (corresponding to $1-2 \times 10^8$ stem cells). According to the present invention, a patient is preferably treated with at least 10^5 CD34+ progenitor cells/kg, e.g. 10^6 to 10^7 CD34+ cells in total, or about 7×10^6 CD34+ progenitor cells for an average male adult human.

The progenitor cells may be administered to a patient in any pharmaceutically or medically acceptable manner, including by injection or transfusion. The cells or supplemented cell populations may be contained, or be contained in any pharmaceutically-acceptable carrier. The cord blood or cord blood-derived stem cells may be carried, stored, or transported in any pharmaceutically or medically acceptable container, for example, a blood bag, transfer bag, plastic tube or vial.

Example 1

Cell source:

Hematopoietic cells are CD34+ progenitor cells (HPC) from umbilical cord blood (UCB) contained within the buffy coat layer.

Collection and processing:

Human umbilical cord blood cells are obtained from umbilical cord blood after normal full-term delivery (informed consent was given). Cord blood is collected into standard 250mL blood bags containing CPD anticoagulant and processed using Sepax (Biosafe) technology within 24 h postpartum for UCB. Cord blood units (buffy coat) are frozen in 10% DMSO using a rate controlled freezer and stored in the liquid phase of liquid nitrogen. Prior to their use, the cells are rapidly thawed (using a 37 degrees C waterbath) into Stemline II serum free media (Sigma, St. Louis, MO, USA) and washed three times in Stemline II media to remove DMSO.

Cell counts, flow cytometry and colony forming assays are performed at this point.

Alginate/Fibronectin/Methylcellulose Encapsulation

Alginate is combined with 0.1% v/v methylcellulose and 50 µg/mL fibronectin.

A total of 10^7 viable cells per chamber are encapsulated in a total of 500 beads i.e. 2.0×10^4 viable cells per bead. Encapsulation media is Stemline II supplemented with Stemsplan CC100 cytokine mix.

Pencillin, streptomycin and gentamycin are used in culture media to prevent the growth of bacterial contaminants in the cord blood unit.

Cord blood buffy coat cells are counted, and resuspended at 1.6×10^6 cells / mL in 0.2µm sterile filtered, at room temperature (21°C), 1.1% (w/v) low viscosity alginic acid (This particular product is a straight-chain, hydrophilic, colloidal, polyuronic acid composed primarily of anhydro-β-D-mannuronic acid residues with 1→4 linkage; Sigma, UK) and 0.1% (v/v) porcine gelatin (Sigma, UK) (all dissolved in PBS, pH 7.4) solution. With a peristaltic pump (Model P-1, Amersham Biosciences, UK), a flow rate producing single droplets, a drop height of 30 mm (tubing autoclaved and then sterilised with 1M NaOH for 30 minutes and washed three times with sterile PBS) the cell-gel solution is passed through the peristaltic pump and dropped using a 25-gauge needle (Becton Dickinson, UK) into sterile, room temperature (21°C), 100 mM calcium chloride solution (CaCl_2 ; Sigma, UK) and 10 mM N-(2-hydroxyethyl) piperazine-N-(2-ethane sulfonic acid) (HEPES; Sigma, UK), in distilled water at pH 7.4. The cell-gel solution gels immediately on contact with the CaCl_2 solution, forming spherical beads (2.3 mm diameter after swelling). The beads remain in gently stirred CaCl_2 solution for 6-10 minutes at room temperature (21°C). The beads are washed three times in PBS and placed into maintenance medium.

The optimum **total** cell count per alginate bead may be in the range of 20,000 cells. It is nevertheless important to ensure that each bead contains an optimum number of CD34+ progenitor cells regardless of total cell count. Cord blood buffy coat cells are therefore assessed for total percentage CD34+ cells in an attempt to standardise the number of CD34+ progenitor cells per bead.

Alginate bead dissolution

A sterile depolymerisation buffer is used to dissolve beads consisting of 50 mM tri-sodium citrate dihydrate (Fluka, UK), 77 mM sodium chloride (BDH Laboratory

supplies, UK) and 10 mM HEPES. The dissolution buffer (Ca^{2+} -depletion) is added to PBS washed beads for 15-20 minutes while stirring gently. The solution is centrifuged at 400g for 10 minutes and the pellet washed with PBS and centrifuged again, at 300g for 3 minutes.

Cytokine combination

The working concentration of the cytokines when used is: 100 ng/mL recombinant human Flt-3 ligand; 100 ng/mL recombinant human Stem Cell Factor; 20 ng/mL recombinant human IL-3; 20 ng/mL recombinant human IL-6.

Seeding Bioreactor

Encapsulated cells are cultured in 50mL of Stemline II media supplemented with Stemspan CC100 cytokine cocktail and antibiotics in a HARV bioreactor. The rotation speed of the bioreactor is 17.5 RPM, and the cells are cultured at 37 degrees C in 5% CO_2 in air.

Feeding encapsulated cells in bioreactor

Day 0 is the day of bioreactor set-up. The bioreactor is fed on day 3, 5 and 7 and harvested on day 10. The old media is aspirated off and 50mL fresh, warm Stemline II media added to the beads, supplemented with Stemspan II cytokine cocktail. The beads are resuspended and reintroduced to the bioreactor.

Harvesting the Bioreactor

The bioreactor is harvested on Day 10. Beads and media are removed from the bioreactor chamber into a centrifuge tube and cells freed by dissolution of the alginate/methylcellulose/fibronectin. The cells are centrifuged at 1500 rpm for 5 mins, the old media decanted off and replaced with fresh media. Cells are assessed using flow cytometry and colony forming unit granulocyte macrophage (CFU-GM) assays. The colony-forming unit-granulocyte-macrophage (CFU-GM) assay is used commonly to assess adequacy of progenitor number in cell populations for bone marrow transplantation.

Analytical Techniques

Morphological assessment: In order to characterize the resulting culture populations, aliquots of cells are deposited on a glass slide (cytocentrifuge, Shandon, Runcorn, UK), fixed and stained in May-Grunwald and Giemsa stain.

CFU-GM assays and CD34+/lin- (lineage negative) flow cytometry are performed using standard procedures according to manufacturer's instructions. CD34+ progenitor cells are identified using a labelled anti-CD34 monoclonal antibody available commercially.

Calculations

Ex vivo expansion of total nucleated cell (TNC), CD34+/lin- cells and CFU are reported either as cumulative numbers; number of cells per ml multiply by the final culture volume, or as fold-expansion ; cumulative numbers divided by initial seeding cell number. CFU frequency is calculated as number of colonies divided by cell number.

Statistics

The following statistical tests are used: The non-parametric test (Wilcoxon Rank Test) is applied for testing differences between the study groups for quantitative parameters. All tests applied are two-tailed, and p value of 5% or less is considered statistically significant. The data is analyzed using the SAS software (SAS Institute).

Example 2

In a further example, the method is performed as in example 1 except that in the flow cytometry step, CD34+/CD133+ is used as a marker combination. An anti-CD133 monoclonal antibody may be used to identify CD133+ cells.

Examples 3 and 4

In further examples, the method is performed as described in a) example 1 and b) example 2 except that the encapsulation medium is PBS instead of Stemline II and no cytokines are added to the medium.

Examples 5 to 8

In further examples, the method is performed as described in each of examples 1 to 4, except that about 200,000 viable cells are encapsulated per bead.

Example 9

In this example, haematopoietic CD34⁺ progenitor cells were expanded using a method similar to that described in Example 1, with the following modifications.

Human cord blood (hCB) mononuclear cells were harvested from hCB units through density gradient Ficoll centrifugation. CD34⁺ progenitor cells were isolated using a magnetic cell sorter (MACS), and only hCB CD34⁺ cells with greater than 95% purity were used. Alternatively CD34⁺ progenitors were obtained frozen from a commercial source.

The isolated hCB CD34⁺ progenitors were encapsulated within a hydrogel comprising 1.1(w/v)% alginate and 1(v/v)% gelatine in PBS, in an expansion medium without serum. The cells were encapsulated at a cell density of 5000 cells per hydrogel bead, each bead having a diameter of approximately 2.5mm. The encapsulated cells were allowed to expand in a NovaPodTM bioreactor system for 10 days.

The CD34⁺ progenitors encapsulated in hydrogel beads at a cell density of 5000 cells/hydrogel were cultured for 10 days and fed on days 0, 4 and 7. The cytokines stem cell factor (SCF), thrombopoietin (TPO) and granulocyte-colony stimulating factor (GCSF) were added to the culture medium (StemLine II), at a concentration of 10 ng/ml at day 0 and 100 ng/ml on days 4 and 7. Two separate experiments under these conditions were performed (A1 and A2 in Table 1), and the results averaged.

A 6.7 fold increase in total cell number was achieved with high cell viability (90%) (see Figures 2 and 3). Encapsulated CD34⁺ progenitors cultured in the same medium but in the absence of cytokines (experiment B in Table 1) showed a lower degree of total cell expansion. After 10 days of expansion, the percentage of CD34⁺ progenitor population decreased from 96% to 76%, as demonstrated by flow cytometry (Figure 1), implying a net 5.3 fold increase (Table 1). At day 10, cells within beads were cultured for a further period without media refresh and allowed to spontaneously differentiate indicating their retention of proliferative potential (Figure 4).

Thus 3D encapsulation and NovaPod bioreactor culture of hCB CD34⁺ progenitors for 10 days, encapsulated in alginate and gelatin in serum-free StemLine II medium, with low concentrations (100ng/mL) of 3 survival cytokines (TPO, G-CSF and SCF) resulted in a 6.7 fold expansion of total nucleated cells and a 5.3 fold increase in CD34⁺ progenitors.

Table 1

Experiment	Media	Expansion of total nucleated cells	Expansion of CD34 ⁺ cells (c.75.72% purity)
A1	StemLine II with 10ng/mL on day 0 in the alginate / cell mixture 100ng/mL SCF, TPO & G-CSF on days 4 and 7	Day 4 = 4600 ± 875 Day 7 = 8145 ± 1055 Day 10 = 36015 ± 4063 10 day expansion = 7.2 fold (Day 0 = 5000, Day 10 = 36015)	10 day expansion = 5.9 fold (Day 0 = 4786.5, Day 10 = 27990.858)
A2	StemLine II with 10ng/mL on day 0 in the alginate / cell mixture 100ng/mL SCF, TPO & G-CSF on days 4 and 7	Day 4 = 4500 ± 912 Day 7 = 8000 ± 714 Day 10 = 31278 ± 1734 10 day expansion = 6.3 fold (Day 0 = 5000, Day 10 = 31278)	10 day expansion = 5.0 fold (Day 0 = 4786.5, Day 10 = 23683.7016)
A (1&2) average	StemLine II with 10ng/mL on day 0 in the alginate / cell mixture 100ng/mL SCF, TPO & G-CSF on days 4 and 7	Day 4 = 4550 ± 830 Day 7 = 8073 ± 838 Day 10 = 33646 ± 3844 10 day expansion = 6.7 fold (Day 0 = 5000, Day 10 = 31278)	10 day expansion = 5.3 fold (Day 0 = 4786.5, Day 10 = 25476.7512)
B	StemLine II only	Day 4 = 2450 ± 957 Day 7 = 3800 ± 849 Day 10 = 5700 ± 572 10 day expansion = 1.14 fold (Day 0 = 5000, Day 10 = 5700)	10 day expansion = 1.1 x decrease (Day 0 = 4786.5, Day 10 = 4316.04)

References

- Andrews, R. G., et al. (1994). *Curr. Opin. Hematol.* 1 (3):187-196.
- Bachier, C. R., et al. (1999). *Exp. Hematol.* 27 (4): 615-623.
- Chabannon, C., et al. (1999a). *Int. J. Oncol.* 15 (3): 511-518.
- Gehling, U. M., et al. (1997). *Exp. Hematol.* 25 (11): 1125-1139.
- Jaroscak, J., et al. *Blood*, 2003 (a).
- Kapelushnik, J., et al. *J Pediatr Hematol Oncol*, 1998 ; 20 (3): 257-9.
- Knutsen, G. , et al. *Tidsskr Nor Laegeforen*, 1998; 118 (16): 2493-7.
- Kurtzberg, J. , et al. *N. Engl. J. Med.* 1996; 335 (3): 157-166.
- Lui, Y., et al (2006a). *Tissue Engineering*, abstract
- Lui, Y., et al (2006b). *J. Biotech* 124, 592-601
- McNiece, I. and R. Briddell *Exp. Hematol.* 2001; 29(1) : 3-11.
- McNiece, I. , et al. *Hematol. Cell Ther.*1999 ; 41 (2):82-86.
- Nielsen, L. K. *Annu Rev Biomed Eng* 1999; 1 : 129-52.
- Noll, T. ,, et al. *Adv Biochem Eng Biotechnol*, 2002 ; 74: 111-28.
- Purdy, M. H., et al. *J. Hematother*, 1995;4 (6): 515-525.
- Shpall, E. J., et al. *Blood* 2000 ; 96 (11 (1)) : 207a.
- Vilquin, J. T., et al. *Arch Mal Coeur Vaiss* 2002 ; 95 (12): 1219-25.
- Wagner, J. E. , Jr. *J. Hematother.*1993 ; 2(2) : 225-228.
- Wagner, J. E. ,, et al. *Blood* 1996 ; 88 (3):795-802.
- Wolff, S. N. *Bone Marrow Transplant* 2002 ; 29 (7): 545-52.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and products of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art are intended to be within the scope of the following claims.

CLAIMS

1. A method of *ex-vivo* expanding umbilical cord blood CD34+ progenitor cells, comprising: (a) encapsulating umbilical cord blood cells in a support matrix; (b) seeding the encapsulated cells into a dynamic culture vessel; and (c) culturing the cells in the dynamic culture vessel under conditions allowing for CD34+ progenitor cell expansion.
2. A method according to claim 1, wherein the cells are derived from human umbilical cord blood.
3. A method according to claim 1 or claim 2, wherein the cells are cultured in a serum free medium.
4. A method according to any preceding claim, wherein the dynamic culture vessel is a bioreactor.
5. A method according to claim 4, wherein the bioreactor is a rotating wall vessel bioreactor.
6. A method according to claim 5, wherein the bioreactor is a HARV bioreactor.
7. A method according to any preceding claim, wherein the support matrix comprises a hydrogel.
8. A method according to any preceding claim, wherein the support matrix comprises alginate.
9. A method according to any preceding claim, wherein the support matrix comprises fibronectin.
10. A method according to any preceding claim, wherein the support matrix comprises methylcellulose.
11. A method according to any preceding claim, wherein the support matrix is in the form of a bead.
12. A method according to any preceding claim, wherein the cells are cultured in a medium comprising one or more cytokines.

13. A method according to claim 12, wherein the cytokines are early acting cytokines.
14. A method according to claim 13, wherein the early acting cytokines are selected from, the group consisting of stem cell factor, FLT3 ligand, interleukin-6 and interleukin-3.
15. A method according to any of claims 1 to 11, wherein the cells are cultured in a cytokine-free medium.
16. A method according to any preceding claim, wherein the cells are cultured in the absence of a transition metal chelator.
17. A method according to any preceding claim, further comprising freezing the encapsulated cells after step (a).
18. A method according to claim 17, further comprising storing the frozen encapsulated cells, and thawing the encapsulated cells before step (b).
19. A method according to any preceding claim, wherein the cells are encapsulated within 72 hours following collection of umbilical cord blood.
20. A method according to any preceding claim, wherein the cells are cultured in the presence of mesenchymal stem cells.
21. A method according to any preceding claim, further comprising releasing the encapsulated cells from the support matrix.
22. An expanded population of umbilical cord blood CD34+ progenitor cells obtainable by a method of any preceding claim.
23. A dynamic culture vessel comprising umbilical cord blood cells encapsulated in a support matrix.
24. A dynamic culture vessel according to claim 23, wherein the dynamic culture vessel is a rotating wall vessel bioreactor.
25. A dynamic culture vessel according to claim 23 or claim 24, wherein the support matrix comprises a hydrogel.

26. A dynamic culture vessel according to any of claims 23 to 25, wherein the umbilical cord blood cells comprise CD34+ progenitor cells.
27. An expanded cell population according to claim 22, for use in regenerative therapy.
28. Use of an expanded cell population according to claim 22, for the preparation of a medicament for regenerative therapy.
29. A method for treating a subject in need of regenerative therapy, comprising administering encapsulated cells according to claim 21 to the subject.
30. A cell population, use or method according to any of claims 27 to 29, wherein the regenerative therapy comprises treating a blood disorder or chemotherapy-associated bone marrow loss.
31. A method comprising (a) encapsulating umbilical cord blood cells in a support matrix; and (b) freezing the encapsulated cells.
32. A method according to claim 1 or any of claims 3 to 21, wherein the cells are obtained from umbilical cord blood from a species of the Equidae family.
33. A method according to claim 12, wherein the cytokines comprise one or more of stem cell factor, thrombopoietin and granulocyte-colony stimulating factor.
34. A method according to claim 11, wherein the cells are encapsulated at a density of 3000 to 300,000 cells per bead.
35. A method according to claim 11 or claim 34, wherein mean diameter of the beads is 1 to 10 mm.
36. A method according to claim 12, wherein each cytokine is present at a concentration of 1 to 100 ng/ml.

1 / 4

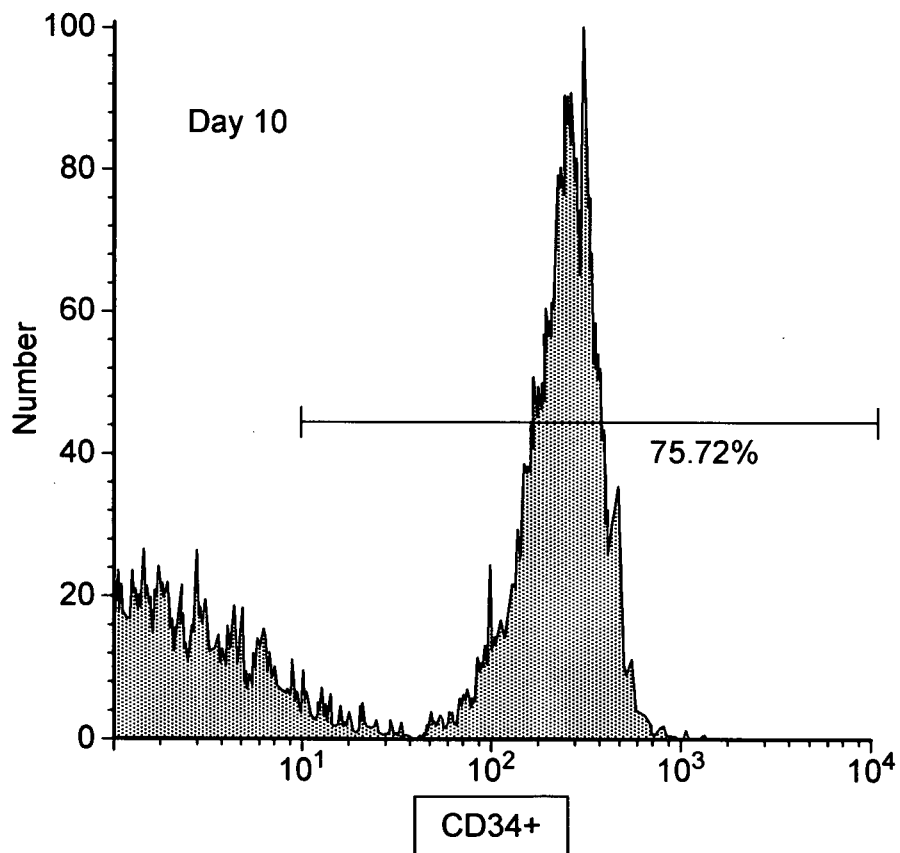
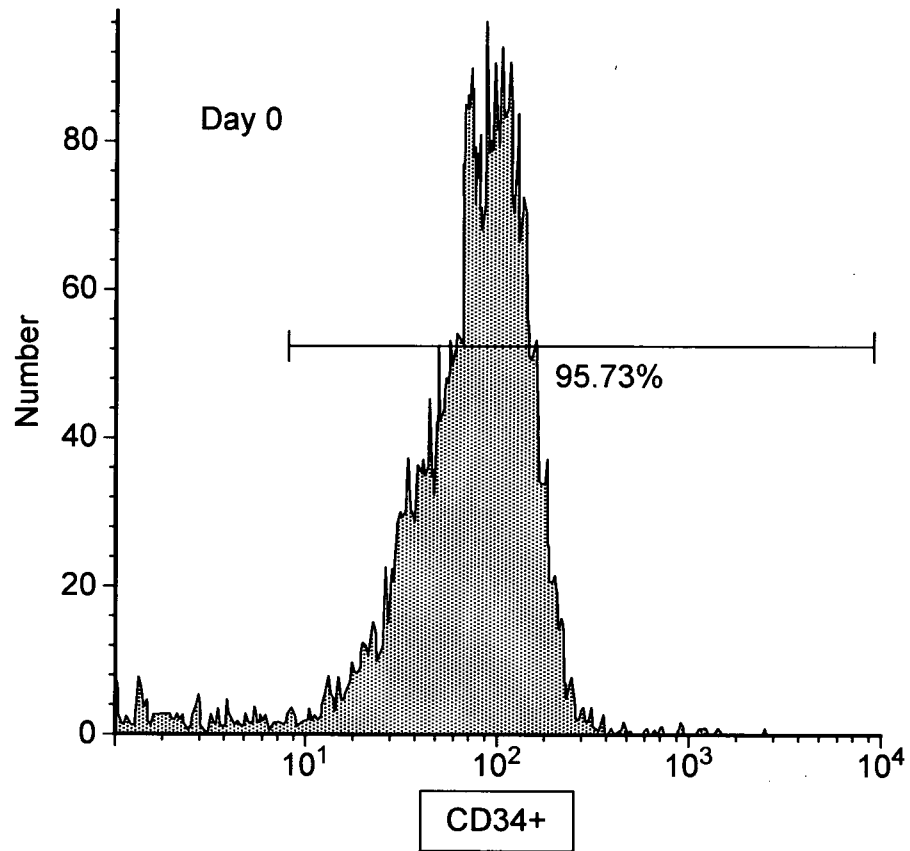


FIG. 1

2 / 4

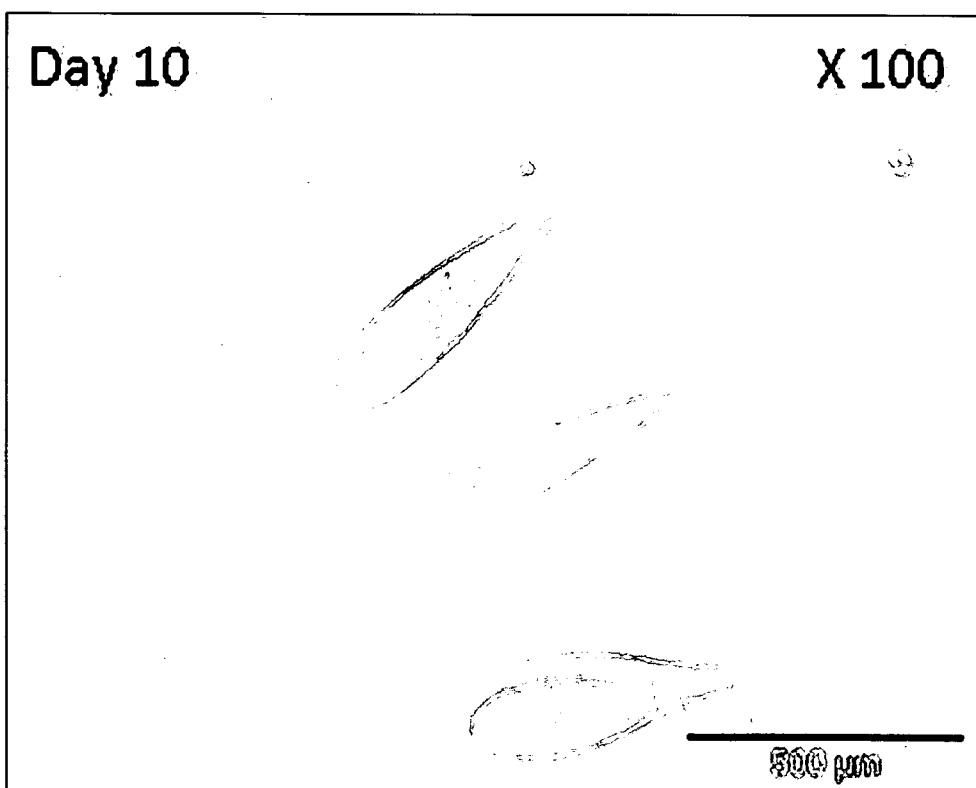
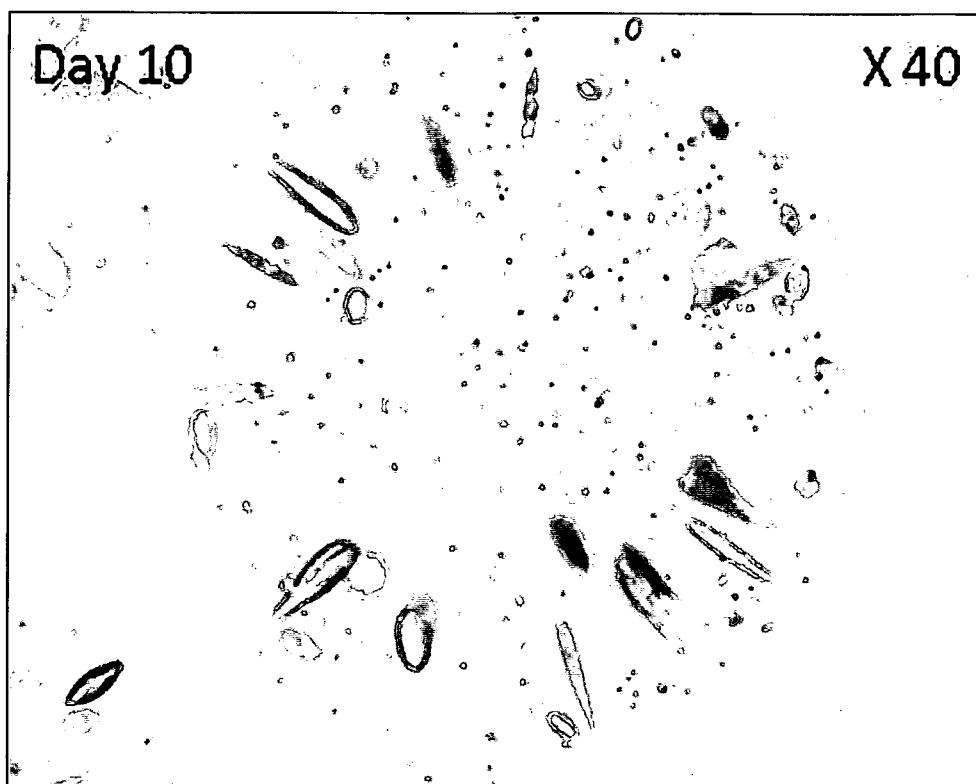


FIG. 2

3 / 4

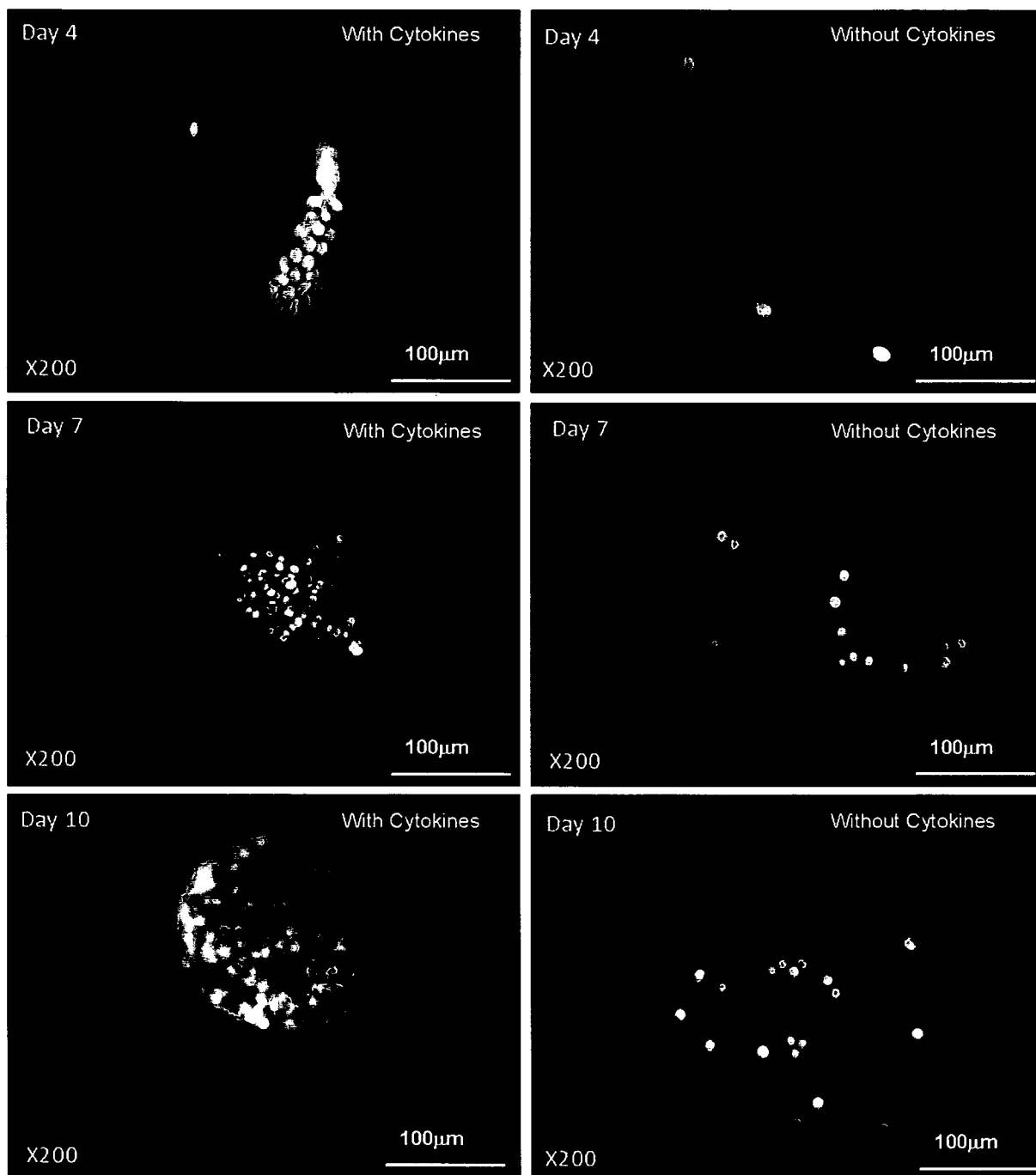


FIG. 3

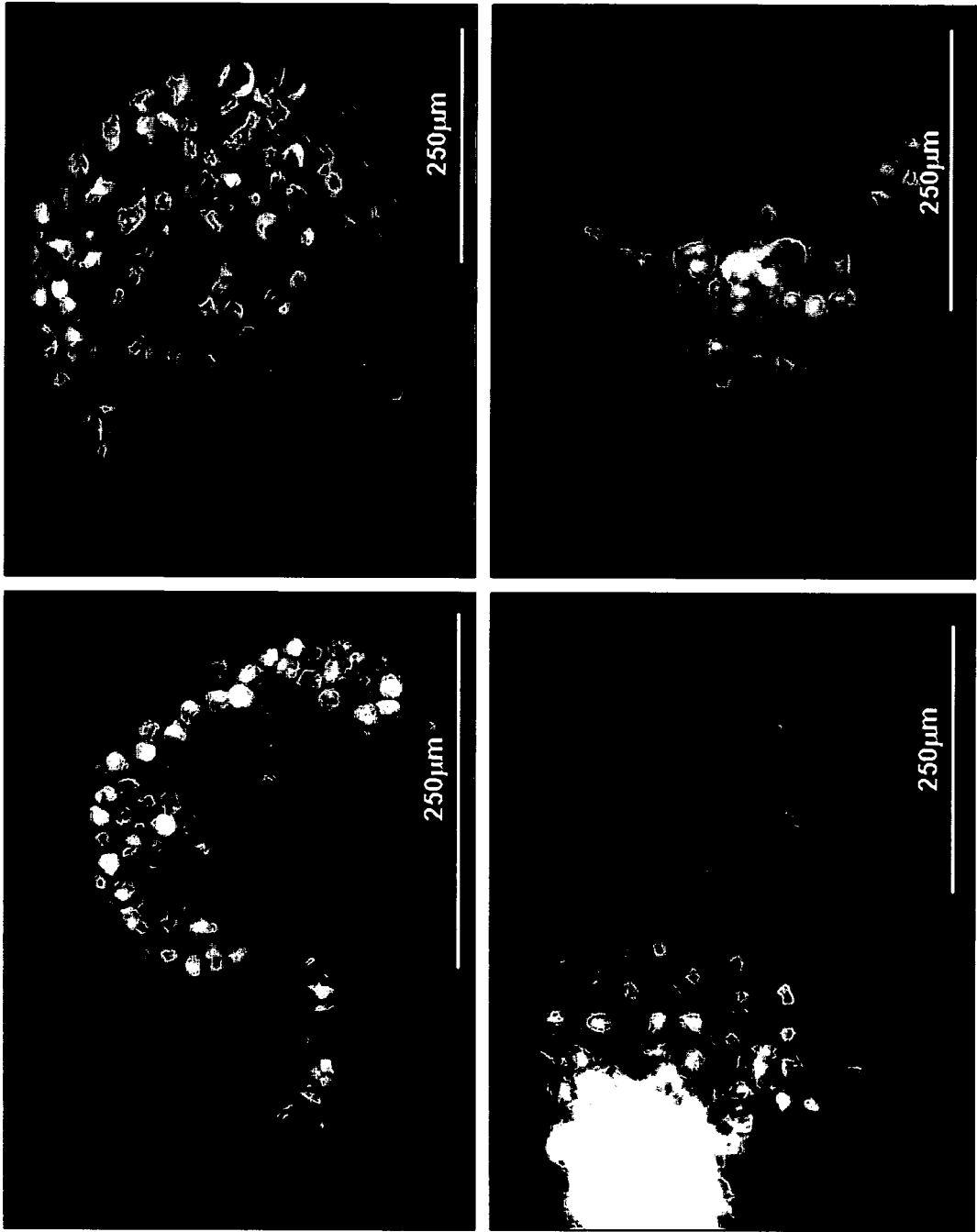


FIG. 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2008/001988

A. CLASSIFICATION OF SUBJECT MATTER
INV. 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)
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, WPI Data, MEDLINE, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2005/007799 A (GAMIDA CELL LTD [IL]; HASSON ARIK [IL]; GRYNSPAN FRIDA [IL]) 27 January 2005 (2005-01-27) cited in the application claims 2,3,9,10,16,19-21 -----	1-36
X	WO 2006/027229 A (RHEINISCHE FRIEDRICH WILHELMS [DE]; TERSTEGGE STEFANIE [DE]; BRUESTLE) 16 March 2006 (2006-03-16)	1-5,9,11
Y	page 3, line 24; claims 12-21,45,57 page 5, line 21 - page 6, line 28 -----	1-36
Y	WO 2006/079854 A (NOVATHERA LTD [GB]; IMP COLLEGE INNOVATIONS LTD [GB]; MANTALARIS SAKIS) 3 August 2006 (2006-08-03) cited in the application page 16, line 27 - line 28 -----	1-36
	-/--	

☒ Further documents are listed in the continuation of Box C.

☒ 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
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- * & * document member of the same patent family

Date of the actual completion of the international search

7 October 2008

Date of mailing of the international search report

27/10/2008

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Nichogiannopoulou, A

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2008/001988

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2006/011855 A (AGENCY SCIENCE TECH & RES [SG]; YU HANRY [US]; TOH YI CHIN [SG] AGENCY) 2 February 2006 (2006-02-02) claims 17,20,24	1-36
P,A	RANDLE WESLEY L ET AL: "Integrated 3-dimensional expansion and osteogenic differentiation of murine embryonic stem cells" TISSUE ENGINEERING, vol. 13, no. 12, December 2007 (2007-12), pages 2957-2970, XP002498656 ISSN: 1076-3279 the whole document	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2008/001988

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2005007799 A	27-01-2005	EP 1649007 A2 US 2006205071 A1	26-04-2006 14-09-2006
WO 2006027229 A	16-03-2006	AU 2005281852 A1 CA 2595137 A1 DE 102004043256 A1 EP 1791950 A1 US 2007264713 A1	16-03-2006 16-03-2006 09-03-2006 06-06-2007 15-11-2007
WO 2006079854 A	03-08-2006	AU 2006208944 A1 CA 2596231 A1 EP 1853698 A1 GB 2437689 A US 2008159994 A1	03-08-2006 03-08-2006 14-11-2007 31-10-2007 03-07-2008
WO 2006011855 A	02-02-2006	EP 1781347 A1 US 2006019362 A1	09-05-2007 26-01-2006