ADHERENT CELLS FROM PLACENTA TISSUE AND USE THEREOF IN THERAPY

RELATED APPLICATIONS:

This application claims the benefit of priority from Provisional Patent Application 61/202,050 filed January 23, 2009 and Provisional Patent Application 61/136,375 filed September 2, 2008.

The contents of all of the above documents are incorporated by reference as if fully set forth herein.

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to adherent cells of placenta tissue and, more particularly, but not exclusively, to methods of culturing same and using same for treatment.

In recent years, considerable activity has focused on the therapeutic potential of mesenchymal stromal cells (MSCs) for various medical applications including tissue repair of damaged organs such as the brain, heart, bone and liver and in support of bone marrow transplantations (BMT). MSCs, a heterogeneous population of cells obtained from e.g. bone marrow, adipose tissue, placenta, and blood, is capable of differentiating into different types of cells (e.g. reticular endothelial cells, fibroblasts, adipocytes, osteogenic precursor cells) depending upon influences from various bioactive factors. Accordingly, MSCs have been widely studied in regenerative medicine as the foundation to build new tissues such as bone, cartilage and fat for the repair of injury or replacement of pathologic tissues and as treatment for genetic and acquired diseases [Fibbe and Noort, Ann N Y Acad Sci (2003) 996: 235-44; Horwitz et al., Cytotherapy (2005) 7(5): 393-5; Zimmet and Hare, Basic Res Cardiol (2005) 100(6): 471-81]. Furthermore, the multipotent ability of MSCs, their easy isolation and culture, as well as their high *ex vivo* expansion potential make them an attractive therapeutic tool [Fibbe and Noort, supra; Minguell et al. Exp Biol Med (Maywood) (2001) 226(6): 507-20].

An emerging body of data indicates that MSCs escape recognition of alloreactive cells and are considered to be immune privileged [Le Blanc et al., Exp Hematol (2003) 31(10): 890-6]. Having low immunogenicity, MSCs are not rejected by the patient's immune system and therefore are considered not to require HLA matching.

Placental derived MSCs exhibit many markers common to MSCs isolated from other tissues, e.g. CD105, CD73, CD90 and CD29, and the lack of expression of hematopoietic, endothelial and trophoblastic-specific cell markers. Adipogenic, osteogenic, and neurogenic differentiation have been achieved after culturing placental derived MSCs under appropriate conditions [Yen et al., Stem Cells (2005) 23(1): 3-9]. Furthermore, MSCs isolated from placenta and cultured *in vitro* have been demonstrated to be immune privileged in a similar fashion as MSCs [Li et al., Cell Res (2005) 15(7): 539-47]. Thus, the placenta provides an ethically non-controversial and easily accessible source of MSCs for experimental and clinical applications [Zhang et al., Exp Hematol (2004) 32(7): 657-64]. In addition, the present inventors have previously devised three dimensional (3D) culturing conditions suitable for expansion of placental derived MSCs (WO/2007/108003).

SUMMARY OF THE INVENTION

In one aspect, there is provided a method of culturing adherent cells from a placenta tissue, the method comprising culturing the adherent cells from the placenta tissue under 3 dimensional (3D) culturing conditions which allow cell expansion, said conditions comprising

perfusion, wherein said perfusion is adjusted according to the glucose concentration of the culture medium,

and

wherein said 3D culturing conditions

(i) comprise a 3D bioreactor;

(ii) comprise an adherent material selected from the group consisting of glass, plastic, polyester, polypropylene, polystyrene, dextran and collagen; and

(iii) are effected for at least 3 days; and

wherein culturing of said cells is effected until at least 10% of said cells are proliferating.

According to some examples of the present disclosure there is provided a method of culturing adherent cells from a placenta or adipose tissue, the method comprising culturing the adherent cells from the placenta or adipose tissue under 3 dimensional (3D) culturing conditions which allow cell expansion, the conditions comprising perfusion.

According to some examples of the present disclosure there is provided a population of cells generated according to the above method.

According to some examples of the present disclosure there is provided a population of cells comprising a gene expression profile essentially as described herein.

According to some examples of the present disclosure there is provided a use of the population of cells, for the manufacture of a medicament identified for treating a condition which can benefit from cell or organ transplantation.

According to some examples of the present disclosure there is provided a method of inducing tolerance and/or immunosuppression in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the adherent cells, thereby inducing tolerance and/or immunosuppression in the subject.

According to some examples of the disclosure, the perfusion is adjusted according to the glucose concentration of the culture medium.

According to some examples of the disclosure, the culture medium is maintained at a glucose concentration of about 550 mg/L.

According to some examples of the disclosure, the 3D culturing conditions comprise a 3D bioreactor.

According to some examples of the disclosure, the 3D culturing conditions comprise an adherent material selected from the group consisting of glass and plastic; polyester, polypropylene, polystyrene, dextran and collagen.

According to some examples of the disclosure, the 3D culturing conditions are effected for at least 3 days.

According to some examples of the disclosure, culturing of the cells is effected until at least 10 % of the cells are proliferating.

According to some examples of the disclosure, at least 10 % of the adherent cells are at a proliferative phase.

According to some examples of the disclosure, the adherent cells are capable of suppressing an immune reaction.

According to some examples of the disclosure, the adherent cells comprise a positive marker expression selected from the group consisting of CD73, CD90, CD29, CD105 and D7-fib.

According to some examples of the disclosure, the adherent cells comprise a negative marker expression selected from the group consisting of CD11b, CD34, HLA-DR, CD14, CD19, CD45, CD31, CD200 and KDR.

According to some examples of the disclosure, the adherent cells comprise a gene expression profile essentially as described herein.

According to some examples of the disclosure, the adherent cells are less committed to an osteogenic lineage as compared to adherent cells from bone marrow grown and allowed to differentiate under the same conditions.

According to some examples of the disclosure, the adherent cells are less committed to an adipogenic lineage as compared to adherent cells from bone marrow grown and allowed to differentiate under the same conditions.

According to some examples of the disclosure, the condition is selected from the group consisting of ischemia, peripheral arterial disease (PAD), critical limb ischemia (CLI), lower extremity ischemia, ischemic vascular disease, vascular disease of the kidney, ischemic heart disease, myocardial ischemia, coronary artery disease (CAD), atherosclerotic cardiovascular disease, left main coronary artery disease, arterial occlusive disease, peripheral ischemia, peripheral vascular disease, arteriosclerosis, ischemic brain disease, stroke, cerebral ischemia, cerebro vascular disease, retinopathy, retinal repair, remodeling disorder, von Hippel-Lindau syndrome, hereditary hemorrhagic telengiectasiaischemic vascular disease, Buerger's disease, ischemic renal disease, ischemic placenta, reproduction associated disorders, graft-versus-host disease, solid organ transplantation, hematopoietic stem cell transplantation, diabetes, connective tissue damage, cancer, pre-cancer, bone cancer, osteosarcoma, bone metastases, bone fracture, burn wound, articular cartilage defect, wound healing, deep wound, delayed wound-healing, delayed ulcer healing, subchondral-bone cyst, osteoporosis, osteoarthritis, degenerated bone, cartilage damage, articular cartilage defect, injured tendons, autoimmune disease, metabolic disorders, psoriasis, neuropathic pain, peripheral nerve injury, support of kidney transplantation and inflammatory disease.

According to some examples of the disclosure, the condition is selected from the group consisting of inflammatory bowel disease (IBD) and Crohn's disease.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the

description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIG. 1 is a flow chart depicting production of 3D adherent cell from placentas according to the present teachings (designated PLX-C cells).

FIG. 2 is a diagram of an exemplary bioreactor vessel and ports adapted from The New Brunswick Scientific web site.

FIGs. 3A-B depict cell cycle analysis of 3D adherent cells manufacture by Plurix (designated PLX, Figure 3B) and by the present teachings (PLX-C, Figure 3A). Cells were fixed in 70 % EtOH O.N, centrifuged and re-suspended in a Propidium Iodide (PI) solution and then analyzed by FACS.

FIGs. 4A-D depict expression of fibroblast-typical markers but not expression of endothelial typical markers on PLX-C. Figure 4A depicts negative expression of the endothelial marker CD31; Figure 4B depicts negative expression of the endothelial marker KDR; Figure 4C depicts positive expression of the human fibroblast marker (D7-FIB), wherein the red histograms for Isotype IgG1 (FITC) represent the negative control while the blue histograms represent the positively stained cells); and Figure 4D depicts negative expression of the human CD200 marker (the pink histograms for Isotype IgG1 (PE) represent the negative control while the green histograms represent the positively stained cells).

FIGs. 5A-D depict expression of stimulatory and co-stimulatory molecules on PLX-C cells. Figure 5A depicts PLX-C expression of CD80; Figure 5B depicts PLX-C expression of CD86; Figure 5C depicts PLX-C expression of CD40; and Figure 5D depicts PLX-C expression of HLA-A/B/C. Negative controls were prepared with relevant isotype fluorescence molecules. Of note, red histograms indicate PLX-C marker-expressing population of cells, blue histograms indicate bone marrow (BM) marker-expressing population of cells, and green histograms indicate mononuclear cell (MNC) marker expressing population of cells.

FIGs. 6A-B depict inhibition of lymphocyte proliferation by PLX-C. Figure 6A depicts Mixed Lymphocyte Reaction (MLR) tests performed with 2 x 10^5 peripheral blood (PB) derived mononuclear cells (MNC, donor A) stimulated with equal amount of irradiated (3000 Rad) PB derived MNCs (donor B) followed by addition of increasing

amounts of PLX-C cells to the cultures. Three replicates of each group were seeded in 96-well plates. Proliferation rate was measured by [^{3}H]thymidine incorporation; Figure 6B depict peripheral blood (PB) derived MNCs stimulated with ConA (1.5 mg /ml). Increasing amounts of PLX-C cells were added to the cultures. Three replicates of each group were seeded in 96-well plates. Proliferation rate was mesured by [^{3}H] thymidine incorporation.

FIGs. 7A-C depict PLX-C regulation of pro-inflammatory and anti-inflammatory cytokine secretion following co-culture with peripheral blood cells. Figures 7A-B depict secretion of IFNγ (Figure 7A) and TNFα (Figure 7B) following co-culture of human derived MNCs (isolated from peripheral blood) stimulated with ConA with PLX-C; Figure 7C depicts secretion of IFNγ, TNFα and IL-10 following co-culture of human derived MNCs (isolated from peripheral blood) stimulated with LPS with PLX-C. Supernatants were collected and subjected to cytokines analysis using ELISA.

FIGs. 8A-F are photographs depicting growth of bone marrow and placenta cells under osteogenesis or adipogenesis differentiation conditions. Bone marrow derived cells (Figures 8A-C) or placenta derived cells (Figures 8D-F) were plated in growth medium (Figures 8A and 8D), osteogenesis differentiation medium (Figures 8B and 8E) or adipogenesis differentiation medium (Figures 8C and 8F) in a 24 well plate coated with vitronectin and collagen. Medium was replaced every 3-4 days. At the end of growth period cells were fixed, stained and pictured as described in detail the Examples section which follows.

FIGs. 9A-F are photographs depicting growth of bone marrow and placenta cells under modified osteogenesis or adipogenesis differentiation conditions. Bone marrow derived cells (Figures 9A-C) or placenta derived cells (Figures 9D-F) were plated in growth medium (Figures 9A and 9D), osteogenesis differentiation medium (Figures 9B and 9E) or adipogenesis differentiation medium (Figures 9C and 9F) in a 24 well plate coated with vitronectin and collagen. Medium was replaced every 3-4 days. At the end of growth period cells were fixed, stained and pictured as described in detail the Examples section which follows.

FIG. 10 depicts the Luciferase expression vector used to infect PLX-C cells. Expression vector Lv33 from OmicsLink was used herein. The Luciferase gene was cloned into the ORF.

FIG. 11 depicts high Luciferase expression by infected PLX-C cells. Cells were infected with the Luciferase expression vector and visualized by the IVIS system 48 hours post infection. Of note, cells exhibited high levels of Luciferase expression.

FIGs. 12A-D depict injection of 2 x 10^6 Luciferase expressing PLX-C cells into SCID/Beige mice. One mouse was injected IM and one IV. The injected mice were monitored using the IVIS system in order to asses the in vivo biodistribution of PLX-C. IVIS results of days 1 (Figure 12A), day 4 (Figure 12B), day 6 (Figure 12C) and day 22 (Figure 12D) are presented.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to adherent cells of placenta tissue and, more particularly, but not exclusively, to methods of culturing same and using same for treatment.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

While reducing the present invention to practice, the present inventors have uncovered that culturing placenta derived adherent cells under three dimensional (3D) culturing conditions, comprising perfusion, generate large amounts of adherent cells which are characterized by a distinctive gene expression profile, are capable of suppressing an immune response and are highly proliferative. Thus, these placenta adherent cells may be used for therapeutic applications.

As is illustrated herein below and in Example 1-8 of the Examples section which follows, the present inventors were able to expand placenta-derived adherent cells under 3D conditions. The 3D conditions of the present invention comprise perfusion of the cell medium within the bioreactor (see Example 2). As is shown in Example 3, the placenta adherent cells of the present invention comprise stromal stem cell properties,

e.g. they express cellular markers typical of stromal stem cells, and comprise immunosuppressive properties. Furthermore, these cells are highly proliferative (28 % of cells were in S and G2/M phases) and are retained in the body for a few weeks after administration (see Examples 3 and 8), suggesting these cells may be used for treatment.

In addition, in their 2D stage, the placenta derived adherent cells of the present invention did not differentiate into osteocytes (Examples 4-5) or adipocytes (Examples 6-7), in sharp contrast to bone marrow adherent cells of which a high percentage (over 50 %) underwent differentiation when grown under the same conditions.

Thus, according to one aspect of the present invention there is provided a method of culturing adherent cells from a placenta or adipose tissue, the method comprising culturing the adherent cells from the placenta or adipose tissue under three dimensional (3D) culturing conditions which allow cell expansion, the conditions comprising perfusion.

As used herein the phrase "adherent cells" refers to a homogeneous or heterogeneous population of cells which are anchorage dependent, i.e., require attachment to a surface in order to grow *in vitro*.

As used herein the phrase "adipose tissue" refers to a connective tissue which comprises fat cells (adipocytes).

As used herein the term "placenta tissue" refers to any portion of the mammalian organ which lines the uterine wall and during pregnancy envelopes the fetus, to which it is attached by the umbilical cord. Following birth, the placenta is expelled (and is referred to as a post partum placenta). In an exemplary embodiment, placenta refers to whole placenta.

According to the present teachings, placenta or adipose tissue derived adherent cells are propagated using three dimensional (3D) culturing conditions.

As used herein the phrase "three dimensional culture" refers to a culture in which the cells are disposed to conditions which are compatible with cell growth including a scaffold which allows cell to cell contacts in three dimensions. It is well appreciated that the *in situ* environment of a cell in a living organism (or a tissue) is in a three dimensional architecture. Cells are surrounded by other cells. They are held in a complex network of extra cellular matrix nanoscale fibers that allows the establishment of various local microenvironments. Their extra cellular ligands mediate not only the

attachment to the basal membrane but also access to a variety of vascular and lymphatic vessels. Oxygen, hormones and nutrients are ferried to cells and waste products are carried away. The conditions in the three dimensional culture of the invention are designed to mimic such an environment as is further exemplified below.

It will be appreciated that the conditions of the three-dimensional culture are such that enable expansion of the adherent cells.

As used herein the terms "expanding" and "expansion" refer to substantially differentiation-less maintenance of the cells and ultimately cell growth, i.e., increase of a cell population (e.g., at least 2 fold) without differentiation accompanying such increase.

As used herein the terms "maintaining" and "maintenance" refer to substantially differentiation-less cell renewal, i.e., substantially stationary cell population without differentiation accompanying such stationarity.

As mentioned, the adherent cells of this aspect of the invention are retrieved from a placental or adipose tissue.

Placental cells may be obtained from a full-term or pre-term placenta. Placenta is preferably collected once it has been ex blooded. The placenta is preferably perfused for a period of time sufficient to remove residual cells (e.g., blood).

The term "perfuse" or "perfusion" used herein refers to the act of pouring or passaging a fluid over the placenta or in the later stages of the method over the cultured cells. The placental tissue may be from any mammal; for example, the placental tissue is human. A convenient source of placental tissue is from a post partum placenta (e.g., 1-6 hours), however, the source of placental tissue or cells or the method of isolation of placental tissue is not critical to the invention.

Placenta derived adherent cells may be obtained from both fetal (*i.e.*, amnion, chorion, chorionic villi or inner parts of the placenta, see Example 1) and maternal (*i.e.*, decidua basalis, and decidua parietalis) parts of the placenta. Tissue specimens are washed in a physiological buffer [e.g., phosphate-buffered saline (PBS) or Hank's buffer]. Single-cell suspensions are made by treating the tissue with a digestive enzyme (see below) or/and mincing and flushing the tissue parts through a nylon filter or by gentle pipetting (Falcon, Becton, Dickinson, San Jose, CA) with washing medium.

It will be appreciated that adherent cells may be derived from adipose tissue. Adipose tissue derived adherent cells may be isolated by a variety of methods known to

those skilled in the art. For example, such methods are described in U.S. Pat. No. 6,153,432. The adipose tissue may be derived from omental/visceral, mammary, gonadal, or other adipose tissue sites. One source of adipose tissue is omental adipose. In humans, the adipose is typically isolated by liposuction.

Isolated adherent cells from placenta or adipose tissue may be derived by treating the tissue with a digestive enzyme such as collagenase, trypsin and/or dispase; and/or effective concentrations of hyaluronidase or DNAse; and ethylenediaminetetra-acetic acid (EDTA); at temperatures between 25 – 50 °C, for periods of between 10 minutes to 3 hours. The cells may then be passed through a nylon or cheesecloth mesh filter of between 20 microns to 1 mm. Cells are centrifuged at speeds of between 100 to 3000 x g for periods of between 1 minutes to 1 hour at temperatures of between 4- 50 °C (see U.S. Pat. No. 7,078,230).

Cell retrieval from placenta or adipose tissue is preferably effected under aseptic conditions. Once isolated cells are obtained, they are allowed to adhere to an adherent material (e.g., configured as a surface) to thereby isolate adherent cells.

As used herein "an adherent material" refers to a synthetic, naturally occurring or a combination of same of a non-cytotoxic (i.e., biologically compatible) material having a chemical structure (e.g., charged surface exposed groups) which may retain the cells on a surface.

Examples of adherent materials which may be used in accordance with this aspect of the invention include, but are not limited to, a polyester, a polypropylene, a polyalkylene, a polyfluorochloroethylene, a polyvinyl chloride, a polystyrene, a polysulfone, a cellulose acetate, a glass fiber, a ceramic particle, a matrigel, an extra cellular matrix component (e.g., fibronectin, vitronectin, chondronectin, laminin), a collagen, a poly L lactic acid, a dextran and an inert metal fiber.

Further steps of purification or enrichment for stromal stem cells may be effected using methods which are well known in the art (such as by FACS using stromal stem cell marker expression, as further described herein below).

Non-limiting examples of base media useful in culturing according to the invention include Minimum Essential Medium Eagle, ADC-1, LPM (Bovine Serum Albumin-free), F10(HAM), F12 (HAM), DCCM1, DCCM2, RPMI 1640, BGJ Medium (with and without Fitton-Jackson Modification), Basal Medium Eagle (BME-with the

addition of Earle's salt base), Dulbecco's Modified Eagle Medium (DMEM-without serum), Yamane, IMEM-20, Glasgow Modification Eagle Medium (GMEM), Leibovitz L-15 Medium, McCoy's 5A Medium, Medium M199 (M199E-with Earle's sale base), Medium M199 (M199H-with Hank's salt base), Minimum Essential Medium Eagle (MEM-E-with Earle's salt base), Minimum Essential Medium Eagle (MEM-H-with Hank's salt base) and Minimum Essential Medium Eagle (MEM-NAA with non essential amino acids), among numerous others, including medium 199, CMRL 1415, CMRL 1969, CMRL 1066, NCTC 135, MB 75261, MAB 8713, DM 145, Williams' G, Neuman & Tytell, Higuchi, MCDB 301, MCDB 202, MCDB 501, MCDB 401, MCDB 411, MDBC 153. A preferred medium for use in the invention is DMEM. These and other useful media are available from GIBCO, Grand Island, N.Y., USA and Biological Industries, Bet HaEmek, Israel, among others. A number of these media are summarized in Methods in Enzymology, Volume LVIII, "Cell Culture", pp. 62 72, edited by William B. Jakoby and Ira H. Pastan, published by Academic Press, Inc.

The medium may be supplemented such as with serum such as fetal serum of bovine or human or other species, and optionally or alternatively, growth factors, vitamins (e.g. ascorbic acid), cytokines, salts (e.g. B-glycerophosphate), steroids (e.g. dexamethasone) and hormones e.g., growth hormone, erythropoeitin, thrombopoietin, interleukin 3, interleukin 6, interleukin 7, macrophage colony stimulating factor, c-kit ligand/stem cell factor, osteoprotegerin ligand, insulin, insulin like growth factors, epidermal growth factor, fibroblast growth factor, nerve growth factor, cilary neurotrophic factor, platelet derived growth factor, and bone morphogenetic protein at concentrations of between picogram/ml to milligram/ml levels.

It is further recognized that additional components may be added to the culture medium. Such components may be antibiotics, antimycotics, albumin, amino acids, and other components known to the art for the culture of cells. Additionally, components may be added to enhance the differentiation process when needed (see further below).

As mentioned, once adherent cells are at hand they may be passaged to two dimensional or three dimensional settings (see Examples 1 and 2 of the Examples section which follows). It will be appreciated though, that the cells may be transferred to a 3D-configured matrix immediately after isolation or alternatively, may be passaged

to three dimensional settings following two dimensional (2D) conditions (as mentioned hereinabove).

It will be appreciated that during the 2D culturing conditions, the adherent cells may be continuously passaged. According to an embodiment of the present invention, the cells may be passaged for at least 4 passages, at least 5 passages, at least 6 passages, at least 7 passages or at least 8 passages. It will be appreciated that cells are typically passaged when the culture reaches about 70-90 % confluence, typically after 3-7 days (e.g., 3-5 days, 1-3 doublings). Moreover, under the 2D culturing conditions, the cells may be grown in a culture medium devoid of antibiotic supplements from at least passage 2, at least passage 3, or at least passage 4.

Thus, the adherent material of this aspect of the invention is configured for 3D culturing thereby providing a growth matrix that substantially increases the available attachment surface for the adherence of the cells so as to mimic the infrastructure of the tissue (e.g., placenta).

For high scale production, culturing can be effected in a 3D bioreactor.

Examples of such bioreactors include, but are not limited to, a plug flow bioreactor, a continuous stirred tank bioreactor, a stationary-bed bioreactor (packed bed bioreactor) and a fluidized bed bioreactor.

As shown in Example 2 of the Examples section, the Celligen bioreactor is capable of 3D expansion of adherent cells under controlled conditions (e.g. pH, temperature and oxygen levels) and with constant cell growth medium perfusion. Furthermore, the cell cultures can be monitored for concentration levels of glucose, lactate, glutamine, glutamate and ammonium. The glucose consumption rate and the lactate formation rate of the adherent cells enable to measure cell growth rate and to determine the harvest time.

Other 3D bioreactors that can be used with the invention include, but are not limited to, a continuous stirred tank bioreactor, where a culture medium is continuously fed into the bioreactor and the used medium is continuously drawn out, to maintain a time-constant steady state within the bioreactor. The stirred tank bioreactor may be used with fluidized bed (suspended carriers) or a fibrous bed basket (which is available for example at New Brunswick Scientific Co., Edison, NJ), a stationary-bed bioreactor, an air-lift bioreactor, where air is typically fed into the bottom of a central draught tube

flowing up while forming bubbles, and disengaging exhaust gas at the top of the column, a bioreactor with Polyactive foams [as described in Wendt, D. et al., Biotechnol Bioeng 84: 205-214, (2003)], a porous scaffolds in a Radial-flow perfusion bioreactor [as described in Kitagawa et al., Biotechnology and Bioengineering 93(5): 947-954 (2006)], a radial flow bioreactor with scaffold or carriers, a hollow fiber bioreactor, and micro carriers. Other bioreactors which can be used in accordance with the invention are described in U.S. Pat. Nos. 6,277,151, 6,197,575, 6,139,578, 6,132,463, 5,902,741 and 5,629,186.

In an exemplary embodiment a total of $150 \pm 50 \times 10^6$ cells are seeded, $3\text{-}7 \times 10^6$ cell / gr carrier are seeded, or $0.06\text{-}0.13 \times 10^6$ cell / ml are seeded. According to an exemplary embodiment, cell seeding is effected at 1400-7000 cells/cm^2 FibraCel disks.

Cells can be harvested when at least about 10 % of cells are proliferating while avoiding uncontrolled differentiation and senescence.

Culturing is effected for at least about 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 10 days, 14 days, 20 days, a month or even more. It will be appreciated that culturing in a bioreactor may prolong this period. Culturing of the adherent cells in the 3D culture can be effected under a continuous flow of a culture medium. Passaging may also be effected to increase cell number. It will be appreciated that culture medium may be changed in order to prolong and improve culturing conditions.

According to an embodiment of the present invention, the cell culturing is effected under perfusion of the culture medium. Typically, the perfusion rate is determined by the glucose concentration in the culture medium of the adherent cells. Thus, according to the present teachings, the culture medium may be changed when the glucose concentration is about 500 mg/L, about 550 mg/L, or about 600 mg/L.

Adherent cells of some embodiments of the present invention comprise at least about 10 %, 28 %, 30 %, 50 %, 80 % or more proliferative cells (as can be assayed by FACS monitoring S and G2/M phases).

Adherent cells of some embodiments of the invention may comprise at least one "stromal stem cell phenotype".

As used herein "a stromal stem cell phenotype" refers to a structural or functional phenotype typical of a bone-marrow derived stromal (i.e., mesenchymal) stem cell.

As used herein the phrase "stem cell" refers to a cell which is not terminally differentiated.

Thus for example, the cells may have a spindle shape. Alternatively or additionally the cells may express a marker or a collection of markers (e.g. surface marker) typical to stromal stem cells. Examples of stromal stem cell surface markers (positive and negative) include but are not limited to CD105+, CD29+, CD44+, CD73+, CD90+, D7-fib+, CD3-, CD4-, CD34-, CD45-, CD80-, CD5-, CD20-, CD11B-, CD14-, CD19-, CD79-, HLA-DR-, CD31-, KDR-, and FMC7-. Other stromal stem cell markers include but are not limited to tyrosine hydroxylase, nestin and H-NF.

Adherent cells of placenta tissue generated according to the present teachings have a gene expression profile essentially as described in Example 3 of the Examples section which follows.

Examples of functional phenotypes typical of stromal stem cells include, but are not limited to, T cell suppression activity (they don't stimulate T cells and conversely suppress same) and hematopoietic stem cell support activity.

According to an exemplary embodiment, the adherent cells of the present invention are less committed to differentiation into osteogenic or adipogenic lineages as compared to adherent cells from the bone marrow grown and differentiated under the same conditions (see Examples 4-5 and Examples 6-7, respectively).

As is shown in Examples 3 of the Examples section which follows, the adherent cells of the present invention were found to suppress the immune reaction of human mononuclear cells in a mixed lymphocyte reaction (MLR) assay, thus exhibit biological activities which may be preferentially used in the clinic (e.g., T cell suppression activity, hematopoietic stem cell support activity).

According to one embodiment of the invention, the adherent cells of the invention are capable of suppressing immune reaction in a subject.

As used herein the phrase "suppressing immune reaction in a subject" refers to decreasing or inhibiting the immune reaction occurring in a subject in response to an antigen (e.g., a foreign cell or a portion thereof). The immune response which can be suppressed by the adherent cells include the humoral immune responses, and cellular immune responses, which involve specific recognition of pathogen antigens via antibodies and T-lymphocytes (proliferation of T cells), respectively.

The populations of cells generated according to the present teachings may be used for treating a condition which can benefit from cell or organ transplantation.

As used herein, the term "condition" refers to any pathology (disease, condition, syndrome or disorder) which may benefit from cell (e.g. stem cell) or organ transplantation. Examples include ischemic conditions, cardiovascular conditions, nervous system conditions, gastrointestinal tract conditions, orthopedic conditions, hematopoietic conditions, renal conditions and hepatic conditions, such as but are not limited to, peripheral arterial disease (PAD), such as limb ischemia and critical limb ischemia (CLI), lower extremity ischemia, ischemic vascular disease, ischemic heart disease, myocardial ischemia, acute myocardial infarction (MI), coronary artery disease (CAD), atherosclerotic cardiovascular disease, left main coronary artery disease, arterial occlusive disease, peripheral ischemia, peripheral vascular disease, arteriosclerosis, ischemic brain disease, stroke, cerebral ischemia, cerebro vascular disease, retinopathy, retinal repair, remodeling disorder, von Hippel-Lindau syndrome, hereditary hemorrhagic telengiectasiaischemic vascular disease, Buerger's disease, diabetes, vascular disease of the kidney, ischemic renal disease, liver disease, ischemic placenta, reproduction associated disorders, graft-versus-host disease (GVHD), solid organ transplant, hematopoietic stem cell transplantation (HSCT), metabolic disorders, inflammatory conditions of the gastrointestinal (GI) tract [e.g. inflammatory bowel disease (IBD)], ulcerative colitis, delayed wound-healing, delayed ulcer healing, cancer (e.g. breast cancer), pre-cancer, conditions characterized by connective tissue damage such as bone cancer, osteosarcoma, bone metastases, bone fracture, degenerative disc disease, osteogenesis imperfecta (OI), burn, burn wound, articular cartilage defect, wound healing, deep wound, delayed wound-healing, delayed ulcer healing, subchondral-bone cyst, osteoporosis, osteoarthritis (OA), degenerated bone, cartilage damage, articular cartilage defect, injured tendons (e.g. overstrain-induced injuries of tendons) and injured ligaments.

It will be appreciated that the adherent cells of the present invention are capable of inducing immunosuppression and/or tolerance in a subject. Thus, the adherent cells may be used to treat any condition in need of immunosuppression and/or tolerance. Such conditions included, but are not limited to, autoimmune diseases and inflammatory diseases (including acute and chronic inflammatory diseases) including,

but are not limited to, cardiovascular diseases, rheumatoid diseases, glandular diseases, gastrointestinal diseases, cutaneous diseases, hepatic diseases, neurological diseases (e.g., neuropathic pain, peripheral nerve injury), muscular diseases, nephric diseases, support for renal transplantation, diseases related to reproduction, connective tissue diseases and systemic diseases.

Examples of autoimmune cardiovascular diseases include, but are not limited to atherosclerosis (Matsuura E. *et al.*, Lupus. 1998;7 Suppl 2:S135), myocardial infarction (Vaarala O. Lupus. 1998;7 Suppl 2:S132), thrombosis (Tincani A. *et al.*, Lupus 1998;7 Suppl 2:S107-9), Wegener's granulomatosis, Takayasu's arteritis, Kawasaki syndrome (Praprotnik S. *et al.*, Wien Klin Wochenschr 2000 Aug 25;112 (15-16):660), anti-factor VIII autoimmune disease (Lacroix-Desmazes S. *et al.*, Semin Thromb Hemost.2000;26 (2):157), necrotizing small vessel vasculitis, microscopic polyangiitis, Churg and Strauss syndrome, pauci-immune focal necrotizing and crescentic glomerulonephritis (Noel LH. Ann Med Interne (Paris). 2000 May;151 (3):178), antiphospholipid syndrome (Flamholz R. *et al.*, J Clin Apheresis 1999;14 (4):171), antibody-induced heart failure (Wallukat G. *et al.*, Am J Cardiol. 1999 Jun 17;83 (12A):75H), thrombocytopenic purpura (Moccia F. Ann Ital Med Int. 1999 Apr-Jun;14 (2):114; Semple JW. *et al.*, Blood 1996 May 15;87 (10):4245), autoimmune hemolytic anemia (Efremov DG. *et al.*, Leuk Lymphoma 1998 Jan;28 (3-4):285; Sallah S. *et al.*, Ann Hematol 1997 Mar;74 (3):139), cardiac autoimmunity in Chagas' disease (Cunha-Neto E. *et al.*, J Clin Invest 1996 Oct 15;98 (8):1709) and anti-helper T lymphocyte autoimmunity (Caporossi AP. *et al.*, Viral Immunol 1998;11 (1):9).

Examples of autoimmune rheumatoid diseases include, but are not limited to rheumatoid arthritis (Krenn V. *et al.*, Histol Histopathol 2000 Jul;15 (3):791; Tisch R, McDevitt HO. Proc Natl Acad Sci units S A 1994 Jan 18;91 (2):437) and ankylosing spondylitis (Jan Voswinkel *et al.*, Arthritis Res 2001; 3 (3): 189).

Examples of autoimmune glandular diseases include, but are not limited to, pancreatic disease, Type I diabetes, thyroid disease, Graves' disease, thyroiditis, spontaneous autoimmune thyroiditis, Hashimoto's thyroiditis, idiopathic myxedema, ovarian autoimmunity, autoimmune anti-sperm infertility, autoimmune prostatitis and Type I autoimmune polyglandular syndrome. diseases include, but are not limited to autoimmune diseases of the pancreas, Type 1 diabetes (Castano L. and Eisenbarth GS.

Ann. Rev. Immunol. 8:647; Zimmet P. Diabetes Res Clin Pract 1996 Oct;34 Suppl:S125), autoimmune thyroid diseases, Graves' disease (Orgiazzi J. Endocrinol Metab Clin North Am 2000 Jun;29 (2):339; Sakata S. *et al.*, Mol Cell Endocrinol 1993 Mar;92 (1):77), spontaneous autoimmune thyroiditis (Braley-Mullen H. and Yu S, J Immunol 2000 Dec 15;165 (12):7262), Hashimoto's thyroiditis (Toyoda N. *et al.*, Nippon Rinsho 1999 Aug;57 (8):1810), idiopathic myxedema (Mitsuma T. Nippon Rinsho. 1999 Aug;57 (8):1759), ovarian autoimmunity (Garza KM. *et al.*, J Reprod Immunol 1998 Feb;37 (2):87), autoimmune anti-sperm infertility (Diekman AB. *et al.*, Am J Reprod Immunol. 2000 Mar;43 (3):134), autoimmune prostatitis (Alexander RB. *et al.*, Urology 1997 Dec;50 (6):893) and Type I autoimmune polyglandular syndrome (Hara T. *et al.*, Blood. 1991 Mar 1;77 (5):1127).

Examples of autoimmune gastrointestinal diseases include, but are not limited to, chronic inflammatory intestinal diseases (Garcia Herola A. *et al.*, Gastroenterol Hepatol. 2000 Jan;23 (1):16), celiac disease (Landau YE. and Shoenfeld Y. Harefuah 2000 Jan 16;138 (2):122), colitis, ileitis and Crohn's disease.

Examples of autoimmune cutaneous diseases include, but are not limited to, autoimmune bullous skin diseases, such as, but are not limited to, pemphigus vulgaris, bullous pemphigoid, psoriasis and pemphigus foliaceus.

Examples of autoimmune hepatic diseases include, but are not limited to, hepatitis, autoimmune chronic active hepatitis (Franco A. *et al.*, Clin Immunol Immunopathol 1990 Mar;54 (3):382), primary biliary cirrhosis (Jones DE. Clin Sci (Colch) 1996 Nov;91 (5):551; Strassburg CP. *et al.*, Eur J Gastroenterol Hepatol. 1999 Jun;11 (6):595) and autoimmune hepatitis (Manns MP. J Hepatol 2000 Aug;33 (2):326).

Examples of autoimmune neurological diseases include, but are not limited to, multiple sclerosis (Cross AH. *et al.*, J Neuroimmunol 2001 Jan 1;112 (1-2):1), Alzheimer's disease (Oron L. *et al.*, J Neural Transm Suppl. 1997;49:77), myasthenia gravis (Infante AJ. And Kraig E, Int Rev Immunol 1999;18 (1-2):83; Oshima M. *et al.*, Eur J Immunol 1990 Dec;20 (12):2563), neuropathies, motor neuropathies (Kornberg AJ. J Clin Neurosci. 2000 May;7 (3):191); Guillain-Barre syndrome and autoimmune neuropathies (Kusunoki S. Am J Med Sci. 2000 Apr;319 (4):234), myasthenia, Lambert-Eaton myasthenic syndrome (Takamori M. Am J Med Sci. 2000 Apr;319

(4):204); paraneoplastic neurological diseases, cerebellar atrophy, paraneoplastic cerebellar atrophy and stiff-man syndrome (Hiemstra HS. *et al.,* Proc Natl Acad Sci units S A 2001 Mar 27;98 (7):3988); non-paraneoplastic stiff man syndrome, progressive cerebellar atrophies, encephalitis, Rasmussen's encephalitis, amyotrophic lateral sclerosis, Sydeham chorea, Gilles de la Tourette syndrome and autoimmune polyendocrinopathies (Antoine JC. and Honnorat J. Rev Neurol (Paris) 2000 Jan;156 (1):23); dysimmune neuropathies (Nobile-Orazio E. *et al.,* Electroencephalogr Clin Neurophysiol Suppl 1999;50:419); acquired neuromyotonia, arthrogryposis multiplex congenita (Vincent A. *et al.,* Ann N Y Acad Sci. 1998 May 13;841:482), neuritis, optic neuritis (Soderstrom M. *et al.,* J Neurol Neurosurg Psychiatry 1994 May;57 (5):544) and neurodegenerative diseases.

Examples of autoimmune muscular diseases include, but are not limited to, myositis, autoimmune myositis and primary Sjogren's syndrome (Feist E. *et al.,* Int Arch Allergy Immunol 2000 Sep;123 (1):92) and smooth muscle autoimmune disease (Zauli D. *et al.,* Biomed Pharmacother 1999 Jun;53 (5-6):234).

Examples of autoimmune nephric diseases include, but are not limited to, nephritis and autoimmune interstitial nephritis (Kelly CJ. J Am Soc Nephrol 1990 Aug;1 (2):140).

Examples of autoimmune diseases related to reproduction include, but are not limited to, repeated fetal loss (Tincani A. *et al.,* Lupus 1998;7 Suppl 2:S107-9).

Examples of autoimmune connective tissue diseases include, but are not limited to, ear diseases, autoimmune ear diseases (Yoo TJ. *et al.,* Cell Immunol 1994 Aug;157 (1):249) and autoimmune diseases of the inner ear (Gloddek B. *et al.,* Ann N Y Acad Sci 1997 Dec 29;830:266).

Examples of autoimmune systemic diseases include, but are not limited to, systemic lupus erythematosus (Erikson J. *et al.,* Immunol Res 1998;17 (1-2):49) and systemic sclerosis (Renaudineau Y. *et al.,* Clin Diagn Lab Immunol. 1999 Mar;6 (2):156); Chan OT. *et al.,* Immunol Rev 1999 Jun;169:107).

Furthermore, the adherent cells of the present invention may be used to treat diseases associated with transplantation of a graft including, but are not limited to, graft rejection, chronic graft rejection, subacute graft rejection, hyperacute graft rejection, acute graft rejection and graft versus host disease.

As used herein the term "treating" refers to inhibiting or arresting the development of a pathology and/or causing the reduction, remission, or regression of a pathology. Those of skill in the art will understand that various methodologies and assays can be used to assess the development of a pathology, and similarly, various methodologies and assays may be used to assess the reduction, remission or regression of a pathology. The term "treating" may also refer to alleviating or diminishing a symptom associated with the pathology.

The subject treated by the adherent cells may be any subject (e.g., a mammal), such as a human subject or a domesticated animal including, but not limited to, horses (i.e. equine), cattle, goat, sheep, pig, dog, cat, camel, alpaca, llama and yak who is diagnosed with or suffers from the pathology and can benefit from stromal stem cell transplantation.

Methods of deriving lineage specific cells from the adherent cells (e.g. stromal stem cells) of the invention are well known in the art. See for example, U.S. Pat. Nos. 5,486,359, 5,942,225, 5,736,396, 5,908,784 and 5,902,741.

The adherent cells may be naïve or may be genetically modified such as to derive a lineage of interest (see U.S. Pat. Appl. No. 20030219423).

The cells may be of autologous or non-autologous source. A non-autologous source may be allogeneic or xenogeneic. Cells may be used as fresh or frozen (e.g., cryo-preserved) preparations.

Depending on the medical condition, the subject may be administered with additional chemical drugs (e.g., immunomodulatory, chemotherapy etc.) or cells.

Even though the cells are characterized by immuno-suppressive activity, they may still provoke host or donor-derived undesirable immune response. Approaches have been developed to reduce the likelihood of rejection of non-autologous cells or GvHD. These include either suppressing the recipient immune system or encapsulating the non-autologous cells in immunoisolating, semipermeable membranes before transplantation.

Encapsulation techniques are generally classified as microencapsulation, involving small spherical vehicles and macroencapsulation, involving larger flat-sheet and hollow-fiber membranes (Uludag, H. et al. Technology of mammalian cell encapsulation. Adv Drug Deliv Rev. 2000; 42: 29-64).

Methods of preparing microcapsules are known in the arts and include for example those disclosed by Lu MZ, et al., Cell encapsulation with alginate and alpha-phenoxycinnamylidene-acetylated poly(allylamine). Biotechnol Bioeng. 2000, 70: 479-83, Chang TM and Prakash S. Procedures for microencapsulation of enzymes, cells and genetically engineered microorganisms. Mol Biotechnol. 2001, 17: 249-60, and Lu MZ, et al., A novel cell encapsulation method using photosensitive poly(allylamine alpha-cyanocinnamylideneacetate). J Microencapsul. 2000, 17: 245-51.

For example, microcapsules are prepared by complexing modified collagen with a ter-polymer shell of 2-hydroxyethyl methylacrylate (HEMA), methacrylic acid (MAA) and methyl methacrylate (MMA), resulting in a capsule thickness of 2-5 μm. Such microcapsules can be further encapsulated with additional 2-5 μm ter-polymer shells in order to impart a negatively charged smooth surface and to minimize plasma protein absorption (Chia, S.M. et al. Multi-layered microcapsules for cell encapsulation Biomaterials. 2002 23: 849-56).

Other microcapsules are based on alginate, a marine polysaccharide (Sambanis, A. Encapsulated islets in diabetes treatment. Diabetes Technol. Ther. 2003, 5: 665-8) or its derivatives. For example, microcapsules can be prepared by the polyelectrolyte complexation between the polyanions sodium alginate and sodium cellulose sulphate with the polycation poly(methylene-co-guanidine) hydrochloride in the presence of calcium chloride.

It will be appreciated that cell encapsulation is improved when smaller capsules are used. Thus, the quality control, mechanical stability, diffusion properties, and *in vitro* activities of encapsulated cells improved when the capsule size was reduced from 1 mm to 400 μm (Canaple L. et al., Improving cell encapsulation through size control. J Biomater Sci Polym Ed. 2002;13:783-96). Moreover, nanoporous biocapsules with well-controlled pore size as small as 7 nm, tailored surface chemistries and precise microarchitectures were found to successfully immunoisolate microenvironments for cells (Williams D. Small is beautiful: microparticle and nanoparticle technology in medical devices. Med Device Technol. 1999, 10: 6-9; Desai, T.A. Microfabrication technology for pancreatic cell encapsulation. Expert Opin Biol Ther. 2002, 2: 633-46).

Examples of immunosuppressive agents include, but are not limited to, methotrexate, cyclophosphamide, cyclosporine, cyclosporin A, chloroquine,

hydroxychloroquine, sulfasalazine (sulphasalazopyrine), gold salts, D-penicillamine, leflunomide, azathioprine, anakinra, infliximab (REMICADE), etanercept, TNFalpha blockers, a biological agent that targets an inflammatory cytokine, and Non-Steroidal Anti-Inflammatory Drug (NSAIDs). Examples of NSAIDs include, but are not limited to acetyl salicylic acid, choline magnesium salicylate, diflunisal, magnesium salicylate, salsalate, sodium salicylate, diclofenac, etodolac, fenoprofen, flurbiprofen, indomethacin, ketoprofen, ketorolac, meclofenamate, naproxen, nabumetone, phenylbutazone, piroxicam, sulindac, tolmetin, acetaminophen, ibuprofen, Cox-2 inhibitors and tramadol.

Furthermore, it will be appreciated that the cells can be administered either *per se* or, preferably as a part of a pharmaceutical composition that further comprises a pharmaceutically acceptable carrier.

As used herein a "pharmaceutical composition" refers to a preparation of the adherent cells of the invention (i.e., adherent cells), with other chemical components such as pharmaceutically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of the cells to a subject.

Hereinafter, the term "pharmaceutically acceptable carrier" refers to a carrier or a diluent that does not cause significant irritation to a subject and does not abrogate the biological activity and properties of the administered compound. Examples, without limitations, of carriers are propylene glycol, saline, emulsions and mixtures of organic solvents with water.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of a compound. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Pharmaceutical compositions for use in accordance with the invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active

ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, physiological salt buffer, or freezing medium containing cryopreservents.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations. However, the amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

Compositions including the preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

Compositions of the invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert.

It is expected that during the life of a patent maturing from this application many relevant three dimensional cultures will be developed and the scope of the term three dimensional cultures is intended to include all such new technologies *a priori*.

As used herein the term "about" refers to ± 10 %.

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of means "including and limited to".

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed.

(1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

Production of placenta derived adherent cells by the methods of WO/2007/108003

Adherent cells were produced as was previously described (see WO/2007/108003) in a bioreactor system containing 3D carriers to produce adherent cells (designated herein as PLX).

Materials and Experimental Procedures

Placenta derived adherent cells - Inner parts of a full-term delivery placenta (Bnei Zion medical center, Haifa, Israel) were cut under aseptic conditions, washed 3 times with Hank's Buffer and incubated for 3 hours at 37 °C with 0.1 % Collagenase (1mg / ml tissue; Sigma- Aldrich, St. Lewis, MO). Using gentle pipetting, suspended cells were then washed with DMEM supplemented with 10 % FCS, Pen-Strep-Nystatin mixture (100 U/ml:100 µg/ml:1.25 un/ml) and 2 mM L-glutamine, seeded in 75 cm^2

flasks and incubated at 37 °C in a tissue culture incubator under humidified condition with 5 % CO_2.

Two dimensional (2D) cell growth

Cells were allowed to adhere to a plastic surface for 48-72 hours after which the media was changed every 3-4 days. After 2-3 passages, the cells were cryopreserved, thawed and seeded for a secondary growth in flasks. When reaching 60-80 % confluence cells were detached from the growth flask using 0.25 % trypsin-EDTA and seeded into new flasks (usually every 3-5 days), for another 2-5 passages. Cultured cells were thereafter collected for analysis or for culturing in bioreactors.

PluriX™ Plug Flow bioreactor - The PluriX™ Plug Flow bioreactor (Pluristem, Haifa, Israel; as was previously described in U.S. Pat. No. 6,911,201 and WO/2007/108003), was loaded with 1-100 ml packed 3D porrosive carriers (4 mm in diameter) made of a non woven fabric matrix of polyester. These carriers enable the propagation of large cell numbers in a relatively small volume. Glassware was designed and manufactured by Pluristem (Pluristem, Haifa, Israel). The bioreactor was maintained in an incubator of 37 °C, with flow rate regulated and monitored by a valve and peristaltic pump. The bioreactor contains a sampling and injection point, allowing the sequential seeding of cells. Culture medium was supplied at pH 6.7-7.4 from a reservoir. The reservoir was supplied by a filtered gas mixture containing air/CO_2/O_2 at differing proportions, depending on cell density in the bioreactor. The O_2 proportion was suited to the level of dissolved O_2 at the bioreactor exit, determined by a monitor. The gas mixture was supplied to the reservoir via silicone tubes or diffuser (Degania Bet, Emek Hayarden, Israel). The culture medium was passed through a separating container which enables collection of circulating, non-adherent cells. Circulation of the medium was obtained by a peristaltic pump. The bioreactor was further equipped with an additional sampling point and containers for continuous medium exchange.

Production of PLX adherent cells - Non-confluent primary human adherent 2D cell cultures, grown as described above, were trypsinized, washed, resuspended in DMEM supplemented with 10 % FBS, Pen-Strep-Nystatin mixture (100 U/ml:100 ug/ml:1.25 un/ml) and 2 mM L-glutamine, and seeded (10^3-10^5 cells/ml) via an injection point onto the 3D carriers in a sterile Plug Flow bioreactor. Prior to

inoculation, bioreactor was filled with PBS-Ca-Mg (Biological Industries, Beit Ha'emek, Israel), autoclaved (120 °C, 30 min) and washed with Dulbecco's growth medium containing 10 % heat-inactivated fetal calf serum and a Pen-Strep-Nystatin mixture (100 U/ml:100 ug/ml:1.25 un/ml). Flow was kept at a rate of 0.1-5 ml/min. Seeding process involved cease of circulation for 2- 48 hrs, thereby allowing the cells to settle on the carriers. Bioreactor was kept under controlled temperature (37 °C) and pH conditions (pH = 6.7-7.4); using an incubator supplied with sterile air and CO_2 as needed. Growth medium was replaced 2-3 times a week. Circulation medium was replaced with fresh DMEM media, every 4 hr to 7 days. At a density of 1 X 10^6-1 X 10^7 cells/ml (following 12-40 days of growth), total medium volume was removed from the bioreactor and bioreactor and carriers were washed 3-5 times with PBS. PLX adherent cells were then detached from the carriers with Trypsin-EDTA; (Biological Industries, Beit Ha'emek, Israel; 3-15 minutes with gentle agitation, 1-5 times), and were thereafter resuspended in DMEM and cryopreserved.

EXAMPLE 2

Production of the placenta derived adherent cells of the present invention

PLX-C adherent cells were produced by the present invention which exhibit different characteristics then the above described PLX adherent cells.

Materials and Experimental Methods

Celligen™ Plug Flow bioreactor – The production of adherent cells of the present invention by Celligen™ (PLX-C cells) is composed of several major steps as illustrated in Figure 1. The process starts by collection of a placenta from a planned caesarean section at term.

Adherent cells are then isolated from whole placentas, grown in tissue culture flasks (2D cultures), harvested and stored in liquid nitrogen as 2D-Cell Stock (2DCS), the appropriate amount of 2DCS are thawed, washed and seeded onto carriers in bioreactors for further expansion as 3D-culture. After 4-12 days of growth in the bioreactors, cells are harvested and cryopreserved in gas phase of liquid nitrogen as PLX-C.

Receipt of Human Tissue

All placentas obtained were received from the maternity ward under approval of the Helsinki Committee of the medical facility. Accordingly, all placenta donors signed an informed consent and Donor Screening and Donor Testing was performed. Immediately after taking the placenta from the donor (during the caesarean procedure), it was placed in a sterile plastic bag and then in a temperature-preserving box with ice packs.

Recovery and Processing of adherent cells

To initiate the process, the placenta tissue was cut into pieces under aseptic conditions under laminar flow hood, washed with Hank's buffer solution and incubated for 2-5 hours at 37 °C with 0.1 % Collagenase (1 mg Collagenase/ml tissue). 2D cell medium (2D-Medium comprising DMEM supplemented with 10 % FBS, fungizone 0.25 μg/ml and Gentamycin 50 μg/ml) was added and the digested tissue was roughly filtered through a sterile metal strainer, collected in a sterile beaker and centrifuged (10 minutes, 1200 RPM, 4 °C). Using gentle pippeting, suspended cells were then diluted with 2D-Medium supplemented with antibiotics, seeded in 175 cm^2 flasks and incubated at 37 °C in a tissue culture incubator under humidified condition supplemented with 5 % CO_2. Following 2-3 days, in which the cells were allowed to adhere to the flask surface, they were washed with PBS and 2D-Medium was added.

Two Dimensional (2D) Cell Growth

Prior to the first passage, growth medium samples of 10 % of the total flask number in quarantine was pooled and taken for mycoplasma testing (IPC2). If cells were found to be negative for Mycoplasma (EZ-PCR Mycoplasma kit, Biological Industries, Israel), cells were released from quarantine. After 1-2 additional passages using 2D-Medium supplemented with antibiotics, cells were transferred to the 2D production clean room (2DP). Once in Room 2DP, culture was continued for another 3-6 passages using 2D-Medium *without* antibiotics. Throughout the process, cultures were grown in a tissue culture incubator under humidified conditions with 5 % CO^2 at 37 °C. After a total of 6-9 passages (9-17 cell doublings), cells were collected and cryopreserved as the 2D-Cell Stock (2DCS).

The first passage was usually carried out after 7-15 days. Beginning at passage 2 and continuing until passage 6-9, cells were passaged when the culture reached 70-90

% confluence, usually after 4-5 days (1.5-2 doublings). The cells were detached from the flasks using 0.25 % trypsin-EDTA (4 minutes at 37 °C) and seeded in a culture density of 4 ± 0.5 x 10^3 cells/cm^2. The size of the tissue culture flasks raised as the passages proceed. The culturing process started in 175 cm^2 tissue culture flask, continued in 500 cm^2 (Triple flask) and finally the cells were seeded into Cell Factory 10 tray (6320 cm^2).

Prior to cryopreservation, at the end of 2DCS growth period, the growth medium was collected and the sample was prepared to be sent to an approved GLP laboratory for Mycoplasma test (IPC 4).

Cryopreservation Procedure for 2D-Cell-Stock Product

For 2DCS cryopreservation, 2D-cultured cells were collected under aseptic conditions using 0.25 % trypsin-EDTA. The cells were centrifuged (1200 RPM, 10', 4 °C), counted and re-suspended in 2D-Medium.

For freezing, cell suspensions were diluted 1:1 with 2D-Freezing Mixture (final concentrations was 10 % DMSO, 40 % FBS and 50 % 2D-Medium). Approximately 1.5 - 2.5 x 10^9 cells were manufactured from one placenta. 4 ml of the cells were stored at a final concentration of 10 x 10^6/ ml in 5 ml cryopreservation polypropylene vials. The vials were labeled and transferred to a controlled rate freezer for a graduated temperature reducing process (1 °C/min), after which they were transferred to storage in gas-phase of a liquid nitrogen freezer. This material was referred to as the 2D-Cell Stock (2DCS) batch.

Initiation of the Three Dimensional (3D) Culture Procedures

To begin 3D culture, an appropriate amount (150 ± 50 x 10^6) of cells from 2DCS were thawed in the 2DP room and washed with 3D-Medium (DMEM with 10 % FBS and 20 Mm Hepes) to remove DMSO prior to seeding in the prepared-in-advanced bioreactor systems. The content of each 2DCS vial was pipetted and diluted 1:9 with pre-warmed (37 °C) 3D-Medium. The cells were centrifuged (1200 RPM, 10', 4 °C) and re-suspended again in 50-100 ml pre-warmed (37 °C) 3D-Medium in a 250 ml sterile bottle. A sample was taken and cells were counted using a Trypan Blue stain in order to determine cell number and viability. The cell suspension was transferred under a laminar flow hood into a 0.5 L seeding bottle. From the seeding bottle the cell suspension was transferred via sterile tubing to the bioreactor by gravitation.

Production of adherent cells in the Celligen Bioreactor (PLX-C)

Bioreactor Description

3D growth phase was performed using an automatic CelliGen Plus® or BIOFLO 310 bioreactor system [(New Brunswick Scientific (NBS)] depicted in Figure 2. The bioreactor system was used for cultivation of cell culture, in which conditions were suitable for high cell concentrations. The cultivation process was carried out using a bioreactor in a perfusion mode. The lab scale bioreactor was constructed of two main systems – the control system and the bioreactor itself (vessel and accessories). The parameters of the process were monitored and controlled by a control console which included connectors for probes, motor and pumps, control loops for Dissolved Oxygen (DO), pH, perfusion and agitation (with a motor), a gases control system, water circulation and heating system for temperature control and an operator interface. The controlled process parameters (such as temperature, pH, DO etc.) could be displayed on the operator interface and monitored by a designated controller.

Cell culture growth procedure in the bioreactors

As noted in the section hereinabove, $150 \pm 50 \times 10^6$ cells from the cryopreserved 2DCS were thawed, washed and seeded in a sterile bioreactor. The bioreactor contained 30-50 gr carriers (FibraCel® disks, NBS), made of Polyester and Polypropylene and 1.5 ± 0.1 L 3D-Medium. The growth medium in the bioreactor was kept at the following conditions: 37 °C, 70 % Dissolved Oxygen (DO) and pH 7.3. Filtered gases (Air, CO_2, N_2 and O_2) were supplied as determined by the control system in order to keep the DO value at 70 % and the pH value at 7.3. For the first 24 hours, the medium was agitated at 50 Rounds Per Minutes (RPM) and increased up to 200 RPM by day 2. For the first 2-3 days, the cells were grown in a batch mode. Perfusion was initiated when the medium glucose concentration decreased below 550 mg/liter. The medium was pumped from the feeding container to the bioreactor using sterile silicone tubing. All tubing connections were performed under laminar flow using sterile connectors. The perfusion was adjusted on a daily basis in order to keep the glucose concentration constant at approximately 550 ± 50 mg\liter. A sample of the growth medium was taken every 1-2 days for glucose, lactate, glutamine, glutamate and ammonium concentration determination (BioProfile 400 analyzer, Nova Biomedical). The glucose consumption rate and the lactate formation rate of the cell culture enabled to measure

cell growth rate. These parameters were used to determine the harvest time based on accumulated experimental data.

Harvest of the 3D Grown PLX-C Cells from the Bioreactor

The cell harvest process started at the end of the growth phase (4-12 days). Two samples of the growth medium were collected. One sample was prepared to be sent to an approved GLP laboratory for Mycoplasma testing according to USP and Eu standards. This medium sample was considered as part of the Mycoplasma testing of the final product and the results were considered as part of the criteria for product release.

The 3D-grown culture was harvested in the Class-100 laminar area in room 3DP as follows:

The bioreactor vessel was emptied using gravitation via tubing to a waste container. The bioreactor vessel was then refilled with 1.5 L pre-warmed PBS (37 °C). The agitation speed was increased to 150 RPM for 2 minutes. The PBS was drained via tubing by pressure or gravity to the waste bottle. The washing procedure was repeated twice.

In order to release the cells from the carriers, 1.5 L pre-warmed to 37 °C Trypsin-EDTA (Trypsin 0.25 %, EDTA 1 mM) was added to the bioreactor vessel and carriers were agitated for 1-4 minutes in 150 RPM, 37 °C. 250 ml FBS was added to the bioreactor vessel and the cell suspension was collected to a 5 L sterile container. Cell suspension was divided to 500 ml sterile centrifuge tubes which were centrifuged (1200 RPM, 10', 4 °C) and re-suspended in cryopreservation solution at a concentration of 5-30^10^6 cells/ml. Cells were aseptically filled and cryopreserved as PLX-C.

EXAMPLE 3

Comparison of the adherent cells of WO/2007/108003 (PLX) to the adherent cells of the present invention

Adherent cells produced by WO/2007/108003 (designated herein as PLX), as described in Example 1 hereinabove, were compared to the new adherent cells of the present invention (designated herein as PLX-C).

Materials and Experimental Methods

Cell Cycle analysis - PLX-C cells obtained by Celligen and PLX cells obtained by Plurix were fixed with 70 % EtOH O.N, centrifuged and re-suspended in a Propidium Iodide (PI) solution containing 2 µg/ml PI (Sigma), 0.2 mg/ml Rnase A (Sigma) and 0.1 % (v/v) Triton (Sigma) for 30 minutes.. Cell cycle was analyzed by FACS.

Gene expression array (Microarray) - Adherent cells were obtained from human full term placentas and were expanded Plurix or by Celligen. Three different batches of cells were obtained from each of the expansion methods for further examination.

RNA was extracted from the cells (Qiagen- Rneasy micro kit) and applied to an Affymetrix whole genome expression array. The chip used GeneChip® Human Exon 1.0 ST Array (Affymetrix, Santa Clara, California, USA).

FACS analysis of membrane markers - cells were stained with monoclonal antibodies as previously described. In short, 400,000-600,000 cells were suspended in 0.1 ml flow cytometer buffer in a 5 ml test tube and incubated for 15minutes at room temperature (RT), in the dark, with each of the following monoclonal antibodies (MAbs): FITC-conjugated anti-human CD29 MAb (eBioscience), PE conjugated anti human CD73 MAb (Becton Dickinson) ,PE conjugated anti human CD105 MAb (eBioscience), PE conjugated anti human CD90 MAb (Becton Dickinson), FITC-conjugated anti-human CD45 MAb (IQProducts), PE-conjugated anti-human CD19 MAb (IQProducts), PE conjugated anti human CD14 MAb (IQProducts), FITC conjugated anti human HLA-DR MAb (IQProduct), PE conjugated anti human CD34 MAb (IQProducts), FITC conjugated anti human CD31 MAb (eBioscience), FITC conjugated anti human KDR MAb (R&D systems), anti human fibroblasts marker (D7-FIB) MAb(ACRIS), FITC-conjugated anti-human CD80 MAb (BD), FITC-conjugated anti-human CD86 MAb (BD), PE conjugated anti-human CD200 MAb (BD), FITC-conjugated anti-human CD40 MAb (BD), FITC-conjugated anti-human HLA-ABC MAb (BD), Isotype IgG1 FITC conjugated (IQ Products), Isotype IgG1 PE conjugated (IQ Products).

Cells were washed twice with flow cytometer buffer, resuspended in 500 µl flow cytometer buffer and analyzed by flow cytometry using FC-500 Flow Cytometer (Beckman Coulter). Negative controls were prepared with relevant isotype fluorescence molecules.

Mixed Lymphocyte Reaction (MLR)

2×10^5 peripheral blood (PB) derived MNC (from donor A) were stimulated with equal amount of irradiated (3000 Rad) PB derived MNCs (from donor B). Increasing amounts of PLX-Cs were added to the cultures. Three replicates of each group were seeded in 96-well plates. Cells were cultured in RPMI 1640 medium containing 20 % FBS. Plates were pulsed with 1 μC ^{3}H-thymidine during the last 18 hrs of the 5-day culturing. Cells were harvested over a fiberglass filter and thymidine uptake was quantified with scintillation counter.

For CFSE staining, PB-MNC cells were stained for CFSE (Molecular Probes) for proliferation measurement before culturing. Cells were collected after 5 days and the intensity of CFSE staining was detected by Flow Cytometry.

ELISA

ELISA was carried out as was previously described. In short, MNCs (isolated from peripheral blood) were stimulated with 5 μg/ml ConA (Sigma), 0.5 μg/ml LPS (SIGMA), or 10 μg/ml PHA (SIGMA) in the presence of PLX-C under humidified 5 % CO2 atmosphere at 37 °C. Supernatants were collected and subjected to cytokine analysis using ELISA kits for IFNγ (DIACLONE), TNFα (DIACLONE) and IL-10 (DIACLONE).

Experimental results

The changes in manufacturing with Celligen as compared to Plurix resulted in several major differences (summarized in Table 1, below).

Table 1: Comparison between Plurix system (WO/2007/108003) and Celligen system (teachings of the present invention)

Parameter	WO/2007/108003	Teachings of the present invention	Improvement
Working volume (ml)	280	1500	Scale up of the process. Higher production level in the present teachings (2-8 population doubling)
Weight of carrier (gr)	1.4	30	Scale up of the process in the

Parameter	WO/2007/108003	Teachings of the present invention	Improvement
			present teachings.
Bed configuration	Conic, 50 ml column	Cylinder Packed bed	The present teachings -- Better flow of medium and nutrients. WO/2007/10800 3 – Inefficient flow due to narrow outlet form the conic structure Better homogeneity of medium flow. Channeling in the plurix
Cell concentration at seeding (cell / gr carrier)	3 x 10^6 cell / gr carrier	5 x 10^6 cell / gr carrier	Better cell to cell interaction in the present teachings
Cell concentration at seeding (cell / ml)	0.015 x 10^6 cell / ml	0.1 x 10^6 cell / ml	Better cell to cell interaction in the present teachings
Seeding procedure	Seeding at low medium volume for 24h followed by addition of medium to final working volume	Seeding at the final working volume while agitating	WO/2007/108003 - Heterogenic distribution of the cell culture inside the carrier bed Insufficient medium volume in the first 24 h of the run. Leading to unsuitable working conditions (acidic environment)
Production phase duration	14-21 days	4-12 days	Better product quality. Efficient harvest process. Better yield. Lower cost process in the present teachings
Mode of operation	Repeated batch – medium change	Perfusion mode – rate	Present teachings - Moderate

Parameter	WO/2007/108003	Teachings of the present invention	Improvement
	twice a week	was adjusted according to the glucose concentration (the medium was changed at glucose concentration of 550 ± 50 mg/L)	changes of the conditions regarding medium composition throughout the run Continuous removal of toxic agents produced by the cells. In batch mode – lower concentration of essential nutrients (limiting factors) Less cell debris
Harvest procedure	Harvesting in 50 ml tubes Trypsinization 3 cycles	Harvesting inside the bioreactor Trypsinization 1 cycle	Present teachings - More efficient process Harvest is carried out in a close system. 1 trypsinization cycle – better quality of the cells.
Agitation	medium Circulation between reservoir container to the column using peristaltic pump	Cell lift impeller	Present teachings - Medium is flowing through the packed bed – Better supply of nutrients and oxygen to the culture. Homogeneity of the medium Improves other control loops (temp., DO, pH)
Temperature control	The production was carried out inside an incubator. Indirect temperature control (of the	On-line direct control. Heat transfer via water jacket.	Present teachings - more accurate measurement of the culture temperature. Quick response. Short time to

Parameter	WO/2007/108003	Teachings of the present invention	Improvement
	incubator chamber). Heat transfer via air interface		reach set point.
Temperature monitoring	Manually. Indirect water temperature monitoring.	On-line direct monitoring.	Present teachings - Better monitoring and control of the process. Quick response to malfunctions.
DO monitoring	None	On-line monitoring	Present teachings - Better monitoring and control of the process. Quick response to malfunctions
DO control	None. Introduction of air only	On-line direct control of a specific set point using Air, O_2 and N_2.	Present teachings - Better control of DO level. Better maintenance of a specified working conditions
pH monitoring and control	Only visual monitoring (Phenol red as part of the medium)	On-line Control and monitoring	Present teachings - Better control of pH level. Better maintenance of a specified working conditions
Aeration	Sparge only	Overlay (sparge as an option)	WO/2007/108003 - Aeration by sparge creates foam that might damage the cells.

The changes in the manufacturing process resulted in changes in characteristics of the obtained adherent cells. These differences are summarized below.

Cell cycle analysis of PLX manufactured by Plurix compared to PLX-C manufactured by Celligen - PLX-C cells obtained by Celligen were compared to PLX cells obtained by Plurix in order to examine the distribution of the cells between the different phases of the cell cycle. As is clear from Figures 3A-B, PLX-C cells expanded by Celligen exhibited typical proliferating profile (distribution of cells between the

different phases of cell cycle). Specifically, 28 % of cells were in S and G2/M phases (Figure 3A). These results indicated that cells were harvested during proliferation and that the Celligen bioreactor conditions supported cell growth.

Microarray comparison between Plurix and Celligen obtained cells - gene expression arrays enabled to simultaneously monitor genome-wide expression profiles of adherent cells derived from human full term placentas expanded by Plurix (PLX) or by Celligen (PLX-C). These results enabled to asses the molecular mechanism underlying phenotypic variation between cells obtained by these different growth methods (see Table 2, below).

Table 2: Gene expression in Plurix cells (WO/2007/108003) compared to Celligen cells (teachings of the present invention)

Gene	Celligen vs Plurix (fold change)	p-value(treat)
interferon-induced protein with tetratricopeptide repeats	**17.52**	0.0401812
aldehyde dehydrogenase 1 family, member A1	**16.76**	0.00145807
leukocyte-derived arginine aminopeptidase	**13.99**	3.88E-06
keratin 27 pseudogene 27	**12.25**	0.000224998
similar to Keratin, type I cytoskeletal 18 (Cytokerati	**11.83**	0.000304949
G protein-coupled receptor, family C, group 5, member A	**10.35**	3.39E-05
integrin, alpha 6	**9.84**	0.0411667
G protein-coupled receptor 126	**8.73**	0.00197635
coagulation factor III (thromboplastin, tissue factor)	**7.36**	0.012192
Rho GDP dissociation inhibitor (GDI) beta	**7.36**	0.00200066
signal peptide, CUB domain, EGF-like 3	**7.20**	0.0255115
interferon-induced protein with tetratricopeptide repeats	**7.09**	0.0139777
dickkopf homolog 1 (Xenopus laevis)	**7.06**	3.06E-07
NAD(P)H dehydrogenase, quinone 1	**6.63**	0.000282423
keratin 18	**6.46**	0.000514523
opioid growth factor receptor-like 1	**5.96**	0.00114551
mal, T-cell differentiation protein-like	**5.95**	0.00664216
neurofilament, medium polypeptide 150kDa	**5.86**	0.0190611
DEP domain containing 1	**5.82**	0.000370513
cathepsin C	**5.72**	0.00532262
WAS	**5.47**	0.00178153
serpin peptidase inhibitor, clade B (ovalbumin), member	**5.44**	0.0190218
solute carrier family 7, (cationic amino acid transporte	**5.33**	0.00688017
interferon-induced protein with tetratricopeptide repea	**5.18**	0.00357376
NUF2, NDC80 kinetochore complex component,	**5.05**	0.00276524

homolog (S. cere		
SHC SH2-domain binding protein 1	4.95	0.00430878
thioredoxin reductase 1	4.86	0.000197486
lung cancer metastasis-associated protein	4.85	0.00148024
Rho GTPase activating protein 29	4.85	0.0466211
cell division cycle 20 homolog (S. cerevisiae)	4.80	0.00514206
family with sequence similarity 111, member B	4.63	0.000125819
PDZ binding kinase	4.54	0.00784983
establishment of cohesion 1 homolog 2 (S. cerevisiae)	4.53	0.000773033
guanylate binding protein 4	4.47	0.000215944
lipase A, lysosomal acid, cholesterol esterase (Wolman dise	4.42	0.0167385
kinesin family member 20A	4.39	0.00582352
KIAA0101	4.28	0.0105909
cyclin-dependent kinase inhibitor 3 (CDK2-associated dual	4.25	0.000732492
thymidylate synthetase	4.23	0.00685584
chromosome 13 open reading frame 3	4.18	0.000548296
aurora kinase A	4.16	0.00632571
nei endonuclease VIII-like 3 (E. coli)	4.14	0.00115606
centrosomal protein 55kDa	4.13	0.0021952
oxidized low density lipoprotein (lectin-like) receptor 1	4.11	0.0205198
denticleless homolog (Drosophila)	4.05	0.00141153
anillin, actin binding protein	4.01	0.010923
ribonucleotide reductase M2 polypeptide	3.98	0.00834059
ankyrin repeat domain 1 (cardiac muscle)	3.93	0.00911953
transcription factor 19 (SC1)	3.89	0.00109627
keratin 18	3.89	0.000112551
non-SMC condensin I complex, subunit G	3.88	0.00537097
cyclin E2	3.87	0.000203389
trypsinogen C	3.86	0.00416276
small nucleolar RNA, C	3.81	0.0334484
tight junction protein 2 (zona occludens 2)	3.81	0.00012562
kinesin family member 18A	3.78	0.00134108
kinesin family member 2C	3.77	0.0059888
shugoshin-like 1 (S. pombe)	3.76	0.00101318
polo-like kinase 1 (Drosophila)	3.75	0.0140309
thymidine kinase 1, soluble	3.73	0.00124134
transcription factor 19 (SC1)	3.73	0.00124327
transcription factor 19 (SC1)	3.73	0.00124327
claspin homolog (Xenopus laevis)	3.71	0.00683624
GINS complex subunit 1 (Psf1 homolog)	3.69	0.00104515
microsomal glutathione S-transferase 1	3.67	0.041701
arylacetamide deacetylase-like 1	3.67	0.000902645
SPC25, NDC80 kinetochore complex component, homolog (S. ce	3.65	0.00568662
integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4	3.62	0.0158411
catenin (cadherin-associated protein), alpha-like 1	3.57	7.46E-05
discs, large homolog 7 (Drosophila)	3.56	0.0317074

v-myb myeloblastosis viral oncogene homolog (avian)-lik	3.55	0.0043878
serglycin	3.54	0.0443487
centromere protein N	3.53	0.000540143
cyclin A2	3.53	0.00965934
heat shock 22kDa protein 8	3.52	0.0219583
sema domain, immunoglobulin domain (Ig), short basic doma	3.49	0.008548
Rho GTPase activating protein 11A	3.49	0.00834174
Fanconi anemia, complementation group I	3.43	0.00464532
BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast	3.42	0.0108258
ovary-specific acidic protein	3.42	0.00334641
cholinergic receptor, muscarinic 2	3.41	0.0320078
cell division cycle 2, G1 to S and G2 to M	3.41	0.0017111
protein regulator of cytokinesis 1	3.39	0.0325664
minichromosome maintenance complex component 5	3.38	0.00475504
sperm associated antigen 5	3.37	0.00906321
maternal embryonic leucine zipper kinase	3.34	0.00908391
small nucleolar RNA, C	3.33	0.0298703
carnitine palmitoyltransferase 1A (liver)	3.33	0.00170894
similar to Ubiquitin-conjugating enzyme E2S (Ubiqui	3.33	0.000415822
kinesin family member 11	3.33	0.00915145
NIMA (never in mitosis gene a)-related kinase 7	3.33	0.00159114
ADAM metallopeptidase with thrombospondin type 1 motif,	3.32	0.0102751
transforming, acidic coiled-coil containing protein 3	3.31	0.0014577
cyclin B1	3.29	0.0103092
MAD2 mitotic arrest deficient-like 1 (yeast)	3.28	0.00488102
dihydrofolate reductase	3.28	0.00178879
NIPA-like domain containing 3	3.27	0.00164708
cell division cycle associated 2	3.26	0.0122226
apolipoprotein B mRNA editing enzyme, catalytic polypep	3.26	0.00308692
cyclin B2	3.25	0.016544
endonuclease domain containing 1	3.24	0.000429245
dihydrofolate reductase pseudogene	3.23	0.00141306
ATPase, Na+	3.23	0.000381464
replication factor C (activator 1) 3, 38kDa	3.23	0.00109668
WD repeat domain 76	3.22	0.0023531
pleckstrin 2	3.17	0.0304429
Rac GTPase activating protein 1	3.17	0.00381613
PHD finger protein 19	3.17	0.000177604
deleted in lymphocytic leukemia, 2	3.15	0.0109528
centromere protein I	3.15	0.0106816
BRCA1 associated RING domain 1	3.14	0.000540414
regulator of G-protein signalling 4	3.13	0.00781061
STAM binding protein-like 1	3.11	0.0181743
sulfiredoxin 1 homolog (S. cerevisiae)	3.10	5.14E-05
chromosome 15 open reading frame 23	3.08	0.000147331
TTK protein kinase	3.08	0.0112171

non-SMC condensin II complex, subunit G2	3.08	0.0130322
villin 2 (ezrin)	3.07	0.0131934
stomatin	3.06	0.00387095
protein tyrosine phosphatase-like A domain containing	3.06	0.0419644
serpin peptidase inhibitor, clade B (ovalbumin), member	3.05	0.0030439
kinesin family member 4A	3.05	0.0114203
hypothetical protein DKFZp762E1312	3.05	0.00726778
ubiquitin-conjugating enzyme E2S	3.04	0.00118205
hydroxysteroid dehydrogenase like 2	3.03	3.71E-05
ATPase family, AAA domain containing 2	3.01	0.00415258
TPX2, microtubule-associated, homolog (Xenopus laevis)	3.00	0.0253137
histone cluster 1, H4d	3.00	0.030183
kinesin family member 23	2.99	0.00790585
heat shock 70kDa protein 2	2.99	0.0215102
origin recognition complex, subunit 1-like (yeast)	2.99	0.00207753
dihydrofolate reductase	2.98	0.00307793
hyaluronan-mediated motility receptor (RHAMM)	2.97	0.00467816
3'-phosphoadenosine 5'-phosphosulfate synthase 2	2.97	1.43E-05
glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	2.95	0.00211969
nucleolar and spindle associated protein 1	2.95	0.00520875
diaphanous homolog 3 (Drosophila)	2.95	0.00107709
kinesin family member 14	2.94	0.00947901
histone cluster 1, H1b	2.93	0.0470898
guanine nucleotide binding protein (G protein), alpha inhi	2.92	0.00184597
minichromosome maintenance complex component 8	2.92	0.000841489
cancer susceptibility candidate 5	2.92	0.0330594
leukotriene B4 12-hydroxydehydrogenase	2.92	0.000685452
glutamate-cysteine ligase, modifier subunit	2.91	0.00378868
forkhead box M1	2.91	0.0203154
adipose differentiation-related protein	2.90	0.000331751
membrane bound O-acyltransferase domain containing 1	2.90	0.01185
ubiquitin-conjugating enzyme E2T (putative)	2.90	0.00741886
cell division cycle associated 3	2.89	0.006289
integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3	2.88	0.00574148
coagulation factor XIII, B polypeptide	2.88	0.0294465
RAD51 homolog (RecA homolog, E. coli) (S. cerevisiae)	2.87	0.000854739
ATP-binding cassette, sub-family C (CFTR	2.87	0.00382491
family with sequence similarity 29, member A	2.85	0.00111165
SH2 domain containing 4A	2.84	0.0323646
membrane protein, palmitoylated 1, 55kDa	2.84	0.000396285
CDC28 protein kinase regulatory subunit 1B	2.84	0.0107391
PSMC3 interacting protein	2.84	0.00766442
elastin microfibril interfacer 2	2.84	0.0192072
topoisomerase (DNA) II alpha 170kDa	2.83	0.0321109
transmembrane protein 106C	2.82	0.000214223

histone cluster 1, H3b	2.80	0.0304598
chromosome 18 open reading frame 24	2.80	0.00347442
epidermal growth factor receptor pathway substrate 8	2.79	0.0194949
high-mobility group nucleosomal binding domain 2	2.78	0.0030536
SCL	2.78	0.00390288
hect domain and RLD 4	2.78	0.00679184
ASF1 anti-silencing function 1 homolog B (S. cerevisiae)	2.77	0.00543408
thyroid hormone receptor interactor 13	2.76	0.0118319
cell division cycle associated 8	2.75	0.00619878
kinesin family member C1	2.74	0.00821937
high-mobility group nucleosomal binding domain 2	2.73	0.00384071
ornithine decarboxylase 1	2.73	0.00144868
v-myb myeloblastosis viral oncogene homolog (avian)-like 2	2.71	0.00989416
KIT ligand	2.70	0.00641955
dual-specificity tyrosine-(Y)-phosphorylation regulated ki	2.70	0.0234606
intraflagellar transport 80 homolog (Chlamydomonas)	2.70	0.0247286
transmembrane protein 48	2.69	0.00458248
EBNA1 binding protein 2	2.69	0.00296292
ZW10 interactor	2.69	1.88E-05
exonuclease 1	2.68	0.00739393
transketolase (Wernicke-Korsakoff syndrome)	2.68	1.92E-05
somatostatin receptor 1	2.68	0.0144901
isocitrate dehydrogenase 3 (NAD+) alpha	2.67	0.00297129
cytoskeleton associated protein 2	2.67	0.0030499
minichromosome maintenance complex component 4	2.67	0.00342054
inhibitor of DNA binding 1, dominant negative helix-loop-hel	2.66	0.036485
CDC28 protein kinase regulatory subunit 1B	2.66	0.0145263
keratin 18	2.66	8.40E-05
CD97 molecule	2.66	0.00994045
chromosome 6 open reading frame 173	2.64	0.00222408
BTB (POZ) domain containing 3	2.62	0.0166824
deafness, autosomal dominant 5	2.62	0.00235481
KIAA0286 protein	2.62	0.00130563
Fanconi anemia, complementation group D2	2.61	0.0281405
polo-like kinase 4 (Drosophila)	2.60	0.00209633
ribonucleotide reductase M1 polypeptide	2.60	0.000170076
malic enzyme 1, NADP(+)-dependent, cytosolic	2.59	0.0435444
non-SMC condensin I complex, subunit H	2.59	0.0216752
S100 calcium binding protein A3	2.58	0.0324073
ubiquitin-conjugating enzyme E2L 3	2.57	0.00343347
BUB1 budding uninhibited by benzimidazoles 1 homolog beta	2.56	0.0166047
glycerol kinase	2.55	2.66E-05
TAF9B RNA polymerase II, TATA box binding protein (TBP)-as	2.54	0.0170365
TAF9B RNA polymerase II, TATA box binding protein (TBP)-as	2.54	0.0170365

histone cluster 1, H2bg	2.52	0.000180822
high-mobility group box 2	2.52	0.0196872
NIMA (never in mitosis gene a)-related kinase 2	2.50	0.00289469
proline rich 11	2.50	0.0357125
myopalladin	2.49	0.0255088
brix domain containing 1	2.49	0.00471977
cell division cycle associated 5	2.49	0.01021
fucosidase, alpha-L- 2, plasma	2.49	0.00540929
cyclin-dependent kinase 2	2.49	0.00250724
lamin B receptor	2.49	0.000151784
hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan synd	2.49	0.000634057
tripartite motif-containing 25	2.47	0.0456344
proteasome (prosome, macropain) subunit, beta type, 9 (lar	2.46	0.0202595
proteasome (prosome, macropain) subunit, beta type, 9 (lar	2.46	0.0202595
proteasome (prosome, macropain) subunit, beta type, 9 (lar	2.46	0.0202595
sphingomyelin synthase 2	2.46	0.0020701
transmembrane protein 62	2.45	0.00761064
glucose-6-phosphate dehydrogenase	2.44	0.00278311
PHD finger protein 1	2.44	0.010191
retinoblastoma-like 1 (p107)	2.44	0.00319946
KIAA1524	2.43	0.0380688
ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,	2.43	0.00830766
cofilin 2 (muscle)	2.43	0.0459235
hypothetical protein LOC201725	2.42	0.000313319
cell division cycle 25 homolog A (S. pombe)	2.42	0.000341692
breast cancer 1, early onset	2.41	0.0180553
transaldolase 1	2.41	0.00199537
mRNA turnover 4 homolog (S. cerevisiae)	2.41	0.00373104
glucosaminyl (N-acetyl) transferase 1, core 2 (beta-1,6-N-	2.41	0.0197148
cysteine rich transmembrane BMP regulator 1 (chordin-like)	2.41	0.0267286
tissue factor pathway inhibitor (lipoprotein-associated	2.40	0.0356227
chromosome 16 open reading frame 59	2.40	0.00185191
glycogenin 1	2.39	0.0224317
transmembrane protein 154	2.39	0.0045589
tubulointerstitial nephritis antigen-like 1	2.39	0.00510812
CTP synthase	2.38	8.80E-05
phenylalanyl-tRNA synthetase, beta subunit	2.38	0.000245973
geminin, DNA replication inhibitor	2.38	0.00167629
lamin B1	2.37	0.0477748
SPC24, NDC80 kinetochore complex component, homolog (S. ce	2.36	0.00287227
glutathione reductase	2.36	0.00353875
ribosomal protein L22-like 1	2.36	0.00335381
fumarylacetoacetate hydrolase (fumarylacetoacetase)	2.36	3.88E-05

small nucleolar RNA, C	2.35	0.0188991
family with sequence similarity 64, member A	2.35	0.0019785
epithelial cell transforming sequence 2 oncogene	2.35	0.000571152
polymerase (DNA directed), epsilon 2 (p59 subunit)	2.34	0.00479612
glycerol kinase	2.34	3.37E-06
glutathione S-transferase M2 (muscle)	2.33	0.0402076
elongation factor, RNA polymerase II, 2	2.33	0.0130017
thioredoxin	2.33	0.009636
polymerase (DNA directed), alpha 2 (70kD subunit)	2.32	0.0033903
breast cancer 2, early onset	2.32	0.00586847
CDC45 cell division cycle 45-like (S. cerevisiae)	2.32	0.00735977
H2A histone family, member Z	2.32	0.0129697
transporter 1, ATP-binding cassette, sub-family B (MDR	2.31	0.0164234
transporter 1, ATP-binding cassette, sub-family B (MDR	2.31	0.0164234
transporter 1, ATP-binding cassette, sub-family B (MDR	2.31	0.0164234
nucleolar complex associated 3 homolog (S. cerevisiae)	2.30	0.000373346
ATPase, Ca++ transporting, plasma membrane 4	2.30	0.023011
minichromosome maintenance complex component 7	2.30	0.0457691
TIMELESS interacting protein	2.29	0.00771062
von Hippel-Lindau binding protein 1	2.28	0.00329061
ras-related C3 botulinum toxin substrate 2 (rho family, sma	2.28	0.0292466
thymopoietin	2.28	0.0223176
peptidylprolyl isomerase F (cyclophilin F)	2.28	0.00093846
activated leukocyte cell adhesion molecule	2.27	0.00242163
polycomb group ring finger 5	2.27	0.000294142
Ran GTPase activating protein 1	2.27	9.68E-05
replication factor C (activator 1) 4, 37kDa	2.26	0.00164152
tubulin, beta 2C	2.26	0.000346744
minichromosome maintenance complex component 10	2.26	0.0037925
H2B histone family, member S	2.25	0.000885505
gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl	2.25	0.0195219
transcription termination factor, RNA polymerase II	2.25	0.000393489
polymerase (DNA directed), delta 2, regulatory subunit 50k	2.25	0.0123823
transporter 1, ATP-binding cassette, sub-family B (MDR	2.25	0.00859077
transporter 1, ATP-binding cassette, sub-family B (MDR	2.25	0.00859077
transporter 1, ATP-binding cassette, sub-family B (MDR	2.25	0.00859077
histone cluster 1, H2bf	2.25	0.0124279
eukaryotic translation initiation factor 1A, X-linked	2.24	0.00330183
phosphoglucomutase 2	2.24	0.00818204
peroxisomal D3,D2-enoyl-CoA isomerase	2.24	0.00148722
interferon-induced protein with tetratricopeptide	2.24	0.0177928

repeats		
G-2 and S-phase expressed 1	2.23	0.0241887
minichromosome maintenance complex component 2	2.23	0.0021347
family with sequence similarity 72, member A	2.23	0.00143248
RMI1, RecQ mediated genome instability 1, homolog (S. cerev	2.23	0.00294705
FLJ20105 protein	2.23	0.0127979
multiple coagulation factor deficiency 2	2.22	0.0116892
phytoceramidase, alkaline	2.22	0.0157729
coiled-coil domain containing 68	2.22	0.00227586
dedicator of cytokinesis 11	2.21	0.00697577
platelet-derived growth factor alpha polypeptide	2.21	0.00176418
N-acylsphingosine amidohydrolase (non-lysosomal cerami	2.20	0.00728536
S-phase kinase-associated protein 2 (p45)	2.20	0.00230153
polymerase (RNA) III (DNA directed) polypeptide G (32kD)	2.20	0.0298794
ADP-ribosylation factor-like 6 interacting protein 1	2.20	0.00139745
histone cluster 1, H2bh	2.19	0.0377748
origin recognition complex, subunit 5-like (yeast)	2.19	0.049697
CDC28 protein kinase regulatory subunit 2	2.19	0.0128024
histone cluster 1, H4c	2.19	0.0112695
hypothetical protein LOC729012	2.19	0.000446087
DEAD (Asp-Glu-Ala-Asp) box polypeptide 39	2.19	0.000340561
chromatin assembly factor 1, subunit B (p60)	2.18	0.0119687
MLF1 interacting protein	2.18	0.0177203
microtubule associated serine	2.18	0.00536974
MHC class I polypeptide-related sequence B	2.18	0.0165406
shugoshin-like 2 (S. pombe)	2.18	0.000852557
COP9 constitutive photomorphogenic homolog subunit 6 (Arab	2.18	0.000793512
methylenetetrahydrofolate dehydrogenase (NADP+ dependent)	2.18	0.00119726
chromosome 6 open reading frame 167	2.18	0.0011095
pituitary tumor-transforming 1	2.17	0.0485166
ribonuclease H2, subunit A	2.17	0.00669936
X-ray repair complementing defective repair in Chinese ham	2.16	0.0369865
membrane protein, palmitoylated 5 (MAGUK p55 subfamily memb	2.16	0.00211873
karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	2.16	0.000650645
pleckstrin homology domain containing, family A (phosphoi	2.15	0.0256434
ribosomal protein L39-like	2.15	0.00429384
karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	2.15	0.000700649
amyloid beta (A4) precursor protein-binding, family B, m	2.15	0.00201004
minichromosome maintenance complex component 3	2.14	0.0018389
histone cluster 1, H2ai	2.14	0.0129155
chromosome 13 open reading frame 34	2.14	0.000702936
RAD18 homolog (S. cerevisiae)	2.14	0.0016685

WD repeat and HMG-box DNA binding protein 1	**2.13**	0.0034833
sulfide quinone reductase-like (yeast)	**2.13**	0.0473641
chromosome 16 open reading frame 63	**2.12**	0.000804179
M-phase phosphoprotein 1	**2.12**	0.0271814
minichromosome maintenance complex component 6	**2.12**	0.0161279
homeobox A9	**2.11**	0.00520942
fibroblast growth factor 9 (glia-activating factor)	**2.10**	0.0475844
cell division cycle 25 homolog C (S. pombe)	**2.10**	0.0169914
chromosome 9 open reading frame 64	**2.10**	0.0265979
U2AF homology motif (UHM) kinase 1	**2.09**	0.0255167
replication factor C (activator 1) 2, 40kDa	**2.09**	0.00768959
hypothetical protein LOC440894	**2.09**	0.0103358
small nuclear ribonucleoprotein D1 polypeptide 16kDa	**2.09**	0.0334665
CSE1 chromosome segregation 1-like (yeast)	**2.09**	0.0013662
phosphatidylinositol glycan anchor biosynthesis, class W	**2.09**	0.0151967
centromere protein O	**2.09**	0.00397056
family with sequence similarity 20, member B	**2.09**	0.00460031
hypothetical protein FLJ40869	**2.09**	0.00444509
guanine nucleotide binding protein (G protein), gamma 11	**2.08**	0.00140559
calcyclin binding protein	**2.08**	0.00524566
ATP-binding cassette, sub-family E (OABP), member 1	**2.08**	0.00454751
CD44 molecule (Indian blood group)	**2.08**	0.000651436
exosome component 8	**2.08**	0.00132017
family with sequence similarity 102, member B	**2.08**	0.025743
histone cluster 2, H3d	**2.07**	0.0102932
family with sequence similarity 33, member A	**2.07**	0.000318673
Fanconi anemia, complementation group B	**2.07**	0.000255109
kinesin family member 22	**2.07**	0.0192406
histone cluster 1, H2ai	**2.07**	0.0161621
vaccinia related kinase 1	**2.06**	0.0233182
integrator complex subunit 7	**2.06**	0.000841371
flap structure-specific endonuclease 1	**2.06**	0.006882
hypothetical protein FLJ25416	**2.06**	0.000177531
ecotropic viral integration site 2B	**2.06**	0.0171408
retinitis pigmentosa 2 (X-linked recessive)	**2.05**	0.0264185
centromere protein L	**2.05**	0.000880856
cofactor required for Sp1 transcriptional activation, subu	**2.04**	0.00141809
chromosome 20 open reading frame 121	**2.04**	0.0146323
family with sequence similarity 72, member A	**2.04**	0.00162905
family with sequence similarity 72, member A	**2.04**	0.00165234
eukaryotic translation initiation factor 1A, X-linked	**2.04**	0.00520549
elongation factor, RNA polymerase II, 2	**2.03**	0.0458007
ATPase, Na+	**2.03**	0.0189108
histone cluster 1, H3a	**2.03**	0.0244273
brix domain containing 1	**2.03**	0.00981178
sushi domain containing 1	**2.03**	0.0258164

ectonucleoside triphosphate diphosphohydrolase 6 (putativ	**2.03**	0.00423628
fructosamine 3 kinase	**2.03**	0.00470972
Bloom syndrome	**2.02**	0.0209259
tubulin, alpha 1c	**2.01**	0.00862586
E2F transcription factor 2	**2.01**	0.0496479
exosome component 2	**2.01**	0.00649147
kinesin family member 22	**2.01**	0.0242075
LTV1 homolog (S. cerevisiae)	**2.01**	0.00812652
dihydrolipoamide S-acetyltransferase (E2 component of pyruv	**2.01**	0.00179011
v-ral simian leukemia viral oncogene homolog B (ras related	**2.01**	0.012225
ring finger and WD repeat domain 3	**2.01**	0.0013797
annexin A1	**2.01**	0.0173578
elaC homolog 2 (E. coli)	**2.00**	0.00266504
aldehyde dehydrogenase 9 family, member A1	**2.00**	0.00911609
tubulin, alpha 4a	**2.00**	0.0435427
nuclear pore complex interacting protein	**-2.00**	0.00111223
oculomedin	**-2.01**	0.00778869
similar to PI-3-kinase-related kinase SMG-1	**-2.01**	0.0356628
golgi autoantigen, golgin subfamily a-like pseudogene	**-2.01**	0.00770626
spectrin repeat containing, nuclear envelope 1	**-2.01**	0.00438469
nuclear pore complex interacting protein	**-2.01**	0.00117582
sushi, nidogen and EGF-like domains 1	**-2.01**	0.00161129
integrin, alpha V (vitronectin receptor, alpha polypeptide	**-2.02**	0.00252702
cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	**-2.04**	0.0150268
lysyl oxidase-like 4	**-2.04**	0.0120148
nuclear pore complex interacting protein	**-2.04**	0.000213956
calcium	**-2.04**	0.00657494
calsyntenin 3	**-2.04**	0.00300887
cell adhesion molecule 1	**-2.05**	0.0261129
solute carrier family 22 (organic cation transporter),	**-2.05**	0.0137275
RUN and FYVE domain containing 3	**-2.05**	0.00387265
glucosidase, alpha; acid (Pompe disease, glycogen storage di	**-2.05**	0.000418401
nuclear pore complex interacting protein	**-2.05**	0.00988632
proline-rich nuclear receptor coactivator 1	**-2.06**	0.0039587
membrane metallo-endopeptidase	**-2.06**	0.0152684
PHD finger protein 21A	**-2.06**	0.00980401
Rho GTPase-activating protein	**-2.06**	0.00705186
homeobox B6	**-2.06**	0.00301714
nuclear pore complex interacting protein	**-2.07**	0.00032839
phospholipase A2 receptor 1, 180kDa	**-2.07**	0.00069343
nuclear pore complex interacting protein	**-2.08**	0.000352007
slit homolog 3 (Drosophila)	**-2.08**	0.02844
nuclear pore complex interacting protein	**-2.09**	0.000414309
cyclin-dependent kinase 6	**-2.09**	0.0456892
dynamin 1	**-2.09**	0.00139674

jumonji, AT rich interactive domain 1B	-2.09	0.00861002
calcium binding and coiled-coil domain 1	-2.09	0.00370041
insulin-like growth factor 1 receptor	-2.09	0.00114467
nuclear pore complex interacting protein	-2.10	0.000377834
CD82 molecule	-2.10	0.0175517
bromodomain adjacent to zinc finger domain, 2B	-2.10	9.88E-05
---	-2.10	0.00666187
synaptotagmin XI	-2.11	0.0129428
KIAA1546	-2.11	0.000255634
jun B proto-oncogene	-2.12	0.0120169
CXXC finger 6	-2.12	0.0277527
nuclear pore complex interacting protein	-2.14	0.00282604
Cdon homolog (mouse)	-2.15	0.0350357
B-cell CLL	-2.15	0.00343507
nuclear pore complex interacting protein	-2.15	0.00263888
v-abl Abelson murine leukemia viral oncogene homolog 1	-2.16	0.0136688
nuclear pore complex interacting protein	-2.16	0.00583397
FAT tumor suppressor homolog 1 (Drosophila)	-2.18	0.0158766
transformer-2 alpha	-2.18	0.012256
chimerin (chimaerin) 1	-2.18	0.0287031
milk fat globule-EGF factor 8 protein	-2.18	0.000987073
vitamin D (1,25- dihydroxyvitamin D3) receptor	-2.19	0.000192208
neuroblastoma, suppression of tumorigenicity 1	-2.20	0.00090639
jumonji domain containing 1A	-2.20	0.0188513
WNK lysine deficient protein kinase 1	-2.21	1.57E-05
protocadherin beta 14	-2.21	0.0103892
cortactin binding protein 2	-2.21	2.28E-05
WW domain containing transcription regulator 1	-2.22	0.0379899
cyclin L1	-2.22	0.00831474
nuclear factor of activated T-cells, cytoplasmic, calcine	-2.22	0.00786451
pellino homolog 1 (Drosophila)	-2.23	0.00939357
golgi autoantigen, golgin subfamily a-like pseudogene	-2.24	0.00603583
chromosome 7 open reading frame 10	-2.26	0.00738442
golgi autoantigen, golgin subfamily a-like pseudogene	-2.27	0.00320764
small Cajal body-specific RNA 17	-2.27	0.0301336
latent transforming growth factor beta binding protein 2	-2.29	4.08E-05
golgi autoantigen, golgin subfamily a, 8A	-2.29	0.0111179
inhibin, beta A (activin A, activin AB alpha polypeptide)	-2.29	0.00877271
solute carrier family 41, member 2	-2.30	0.00453672
forkhead box P1	-2.30	0.0463138
matrix metallopeptidase 14 (membrane-inserted)	-2.31	1.93E-05
transcription factor 4	-2.31	0.0367869
jun oncogene	-2.32	7.21E-05
neuroepithelial cell transforming gene 1	-2.33	0.0109689
asporin	-2.33	0.000659873
v-fos FBJ murine osteosarcoma viral oncogene homolog	-2.35	0.0138624

ephrin-B2	-2.36	0.00611474
WD repeat and SOCS box-containing 1	-2.36	0.0387851
similar to dJ402H5.2 (novel protein similar to wo	-2.36	0.00621503
PX domain containing serine	-2.38	0.000927628
collagen, type VII, alpha 1 (epidermolysis bullosa, dystr	-2.38	0.00109233
AE binding protein 1	-2.39	0.000105628
peroxidasin homolog (Drosophila)	-2.40	0.00219049
calcium channel, voltage-dependent, L type, alpha 1C sub	-2.41	0.0189661
Prader-Willi syndrome chromosome region 1	-2.45	0.0415526
midline 1 (Opitz	-2.45	0.00130803
nuclear pore complex interacting protein	-2.45	0.00354416
chromosome 1 open reading frame 54	-2.47	0.0186089
transmembrane protein 16A	-2.48	0.0481085
basic helix-loop-helix domain containing, class B, 2	-2.49	0.00270257
nuclear pore complex interacting protein	-2.50	0.00316496
runt-related transcription factor 1 (acute myeloid leukemi	-2.50	0.000607387
zinc finger protein 292	-2.50	0.029832
fibronectin leucine rich transmembrane protein 2	-2.51	0.0135122
nuclear pore complex interacting protein	-2.51	0.00283418
potassium voltage-gated channel, subfamily G, member 1	-2.54	0.0244306
interleukin 19	-2.54	0.0310328
transforming growth factor, beta 3	-2.54	0.0287865
dihydropyrimidinase-like 3	-2.55	0.0165203
golgi autoantigen, golgin subfamily a, 8B	-2.56	0.0121417
hypothetical protein PRO2012	-2.57	0.00756704
SATB homeobox 2	-2.57	0.039781
t-complex 11 (mouse)-like 2	-2.57	0.0324227
ring finger protein 122	-2.57	0.0236621
chromosome 8 open reading frame 57	-2.59	0.00261522
ADAM metallopeptidase with thrombospondin type 1 motif,	-2.60	0.0113968
sushi, von Willebrand factor type A, EGF and pentraxin dom	-2.63	2.23E-05
ST6 beta-galactosamide alpha-2,6-sialyltranferase 2	-2.64	0.0216987
sortilin-related VPS10 domain containing receptor 2	-2.65	0.00936311
protocadherin beta 9	-2.66	0.0285124
chromosome 5 open reading frame 13	-2.67	0.00410172
Enah	-2.68	0.0077547
pyridoxal-dependent decarboxylase domain containing 2	-2.69	0.00683647
similar to nuclear pore complex interacting protein	-2.70	0.0187322
nuclear pore complex interacting protein	-2.70	0.00368967
transmembrane protein 119	-2.70	0.00801387
chromosome 14 open reading frame 37	-2.70	0.0182453
sushi-repeat-containing protein, X-linked 2	-2.71	0.0253856
PDZ domain containing RING finger 3	-2.71	0.00931014
collagen, type XII, alpha 1	-2.72	0.000204664

matrix-remodelling associated 5	-2.72	0.000317637
collagen, type V, alpha 1	-2.72	0.0166427
dystrophin related protein 2	-2.72	0.0137557
ATP-binding cassette, sub-family A (ABC1), member 1	-2.73	0.00131361
trophinin	-2.77	0.00298044
cornichon homolog 3 (Drosophila)	-2.78	0.0261738
formin binding protein 1-like	-2.78	0.00290401
brain and acute leukemia, cytoplasmic	-2.78	0.0476919
protein tyrosine phosphatase, receptor type, U	-2.80	0.0270428
hypothetical protein MGC24103	-2.82	0.0346673
interferon induced with helicase C domain 1	-2.83	0.0024839
phospholipid transfer protein	-2.84	0.00999206
immediate early response 3	-2.87	0.0152127
immediate early response 3	-2.87	0.0152127
ADAM metallopeptidase domain 12 (meltrin alpha)	-2.87	0.000870288
synaptic vesicle glycoprotein 2A	-2.88	0.00704212
chromosome 9 open reading frame 3	-2.88	0.00410177
thioredoxin interacting protein	-2.90	0.0135494
early growth response 1	-2.93	0.000425035
small nucleolar RNA, C	-2.94	0.00666866
small nucleolar RNA, C	-2.95	0.00765575
immediate early response 3	-2.99	0.0167309
low density lipoprotein-related protein 1 (alpha-2-macroglo	-2.99	4.26E-05
bicaudal C homolog 1 (Drosophila)	-2.99	0.0347162
homeobox B2	-3.03	0.00665994
small nucleolar RNA, C	-3.10	0.0274043
small nucleolar RNA, C	-3.10	0.0274043
matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase,	-3.13	5.59E-05
KIAA1641	-3.14	0.00659194
collagen, type VI, alpha 3	-3.14	2.09E-06
homeobox A2	-3.15	0.0435423
SH3 and PX domains 2B	-3.15	0.0244357
collagen, type VI, alpha 2	-3.16	0.0149554
chromosome 9 open reading frame 3	-3.21	0.0233723
small nucleolar RNA, C	-3.24	0.0104491
small nucleolar RNA, C	-3.24	0.0104491
---	-3.27	0.00488845
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylga	-3.35	0.00964109
cholesterol 25-hydroxylase	-3.38	0.0445558
KIAA1641	-3.40	0.013175
ring finger protein 144	-3.40	0.0135334
versican	-3.41	0.023885
angiopoietin-like 2	-3.42	0.0245161
KIAA1641	-3.44	0.0170531
FBJ murine osteosarcoma viral oncogene homolog B	-3.54	0.00025573
similar to RIKEN cDNA 1110018M03	-3.59	0.00516476
early growth response 2 (Krox-20 homolog,	-3.62	0.00821813

Drosophila)		
dachsous 1 (Drosophila)	-3.63	0.00697244
kinesin family member 26B	-3.64	0.00363199
distal-less homeobox 5	-3.66	0.000640157
similar to Protein KIAA0220	-3.69	0.0302619
insulin-like growth factor 1 receptor	-3.71	3.42E-05
protein tyrosine phosphatase, receptor type, N	-3.77	0.0294569
KIAA1641	-3.85	0.0191782
sushi-repeat-containing protein, X-linked	-3.85	0.00370941
microfibrillar-associated protein 2	-3.91	0.0152901
complement component 1, s subcomponent	-3.97	0.0395863
CD24 molecule	-3.99	0.0340122
homeobox B3	-4.02	0.0354368
trichorhinophalangeal syndrome I	-4.02	0.00557712
Kallmann syndrome 1 sequence	-4.04	0.000548703
leucine rich repeat containing 17	-4.09	0.0263961
plexin domain containing 2	-4.32	0.031799
PTK7 protein tyrosine kinase 7	-4.42	0.000116114
supervillin	-4.43	0.0412717
zinc finger protein 521	-4.58	0.00668815
calbindin 2, 29kDa (calretinin)	-4.77	0.0290743
ras homolog gene family, member J	-4.79	0.00197982
integrin, alpha 11	-4.80	0.000390317
odz, odd Oz	-5.05	0.00172671
F-box protein 32	-5.52	0.0212957
raftlin family member 2	-5.72	0.0260454
clusterin	-5.74	0.0303973
neurotrimin	-5.79	3.78E-06
WNT1 inducible signaling pathway protein 1	-5.86	0.000672342
insulin-like growth factor binding protein 5	-6.34	0.011614
sulfatase 2	-6.34	5.88E-05
microfibrillar-associated protein 4	-6.93	0.00155578
junctional adhesion molecule 2	-7.07	0.0306758
fibronectin type III domain containing 1	-7.29	0.0334696
sarcoglycan, delta (35kDa dystrophin-associated glycoprotei	-7.37	0.000881984
hephaestin	-7.53	0.0123141
serpin peptidase inhibitor, clade F (alpha-2 antiplasmi	-7.66	0.00362941
cystatin SN	-7.96	0.0496433
hemicentin 1	-8.18	0.0461603
tenascin C (hexabrachion)	-8.32	8.26E-05
biglycan	-8.62	0.00161284
transmembrane, prostate androgen induced RNA	-11.20	0.000100935
carboxypeptidase E	-11.22	0.00738131

Expression of cellular markers on PLX-C cells - the surface antigens expressed by PLX-C were examined using monoclonal antibodies. Results indicated that PLX-C

cells were characterized by the positive markers: CD73, CD29 and CD105 and the negative markers: CD34, CD45, CD19, CD14, CD200 and HLA-DR. In some experiments, the immune phenotype test specifications were set as: $\geq$ 90 % for all positive markers and $\leq$ 3 % for all negative markers.

Furthermore, as shown in Figures 4A-B, PLX-C cultures did not express endothelial markers as shown by negative staining for the two endothelial markers CD31 and KDR. However, PLX-C expression of a fibroblast-typical marker was evident (expression of D7-fib, Figure 4C). In addition, as is shown in Figure 4D, PLX-C cells negatively express CD200.

Immunogenecity and immunomodulatory properties of PLX-C cells- as PLX-C is comprised of adherent cells derived from placenta, it is expected to express HLA type I, which is expressed by all cells of the body and is known to induce an alloreactive immune response. HLA type II and other co-stimulatory molecules are typically expressed only on the surface of Antigen Presenting Cells (APCs).

In order to examine the immunogenicity of the obtained PLX-C cells, the expression of co-stimulatory molecules on the surface of these cell membranes were performed. FACS analysis demonstrated the absence of CD80, CD86 and CD40 on the PLX-C cell membranes (Figures 5A-C). Moreover, PLX-C expressed low levels HLA class I as detected by staining for HLA A/B/C (Figure 5D). The expression of stimulatory and co-stimulatory molecules was similar to bone marrow (BM) derived MSCs (as shown in Figures 5A-D).

To further investigate the immunogenecity as well as the immunomodulation properties of PLX-C cells, Mix Lymphocyte Reaction (MLR) tests were performed. As shown in Figure 6A-B, PLX-C cells both escape allorecognition and reduce T cell response, as measured by Thymidine incorporation. Furthermore, the reduction in lymphocytes proliferation (evaluated by CPM measurement) was higher as the number of PLX-C cells increased (in a dose dependent manner). PLX-C also reduced lymphocyte proliferation following mitogenic stimuli, such as Concavalin A (Con A, Figure 6B) and Phytohemagglutinin (PHA), and non-specific stimulation by anti-CD3, anti-CD28 (data not shown).

In order to investigate the mechanism of action by which PLX-C immunomodulate lymphocyte proliferation, and to see if this action is mediated via cell

to cell interaction or cytokines secretion, PB derived Mononuclear cells (MNCs) were stimulated by PHA using the transwell method (which prevents cell to cell contact but enables the diffusion of cytokines between the two compartments). Results showed that the inhibition of proliferation maintained even when cell to cell contact was inhibited (data not shown).

Cytokines secretion – as depicted hereinabove, PLX-C reduce the proliferation rate of lymphocytes, probably through soluble factors. Further investigation of the cytokines secreted by lymphocytes in response to PLX-C was performed to elucidate the mechanism of action of PLX-C. As depicted in Figures 7A-B, culturing of mononuclear cells with PLX-C slightly reduces the secretion of the pro-inflammatory cytokine INFγ and dramatically reduces the secretion of TNFα (even in the presence of low amounts of PLX-C). In addition, following lipopolysaccharide (LPS) stimulation, PB derived MNCs secretion of IL-10 increased in the presence of PLX-C, while the secretion level of TNFα decreased, in a dose dependent manner (Figure 7C).

EXAMPLE 4

Comparison of Osteocyte differentiation of 2D adherent cells of the present invention and bone marrow cells

The new adherent cells of the present invention (from placenta origin) were grown under osteocyte differentiation stimulating conditions at their 2D adherent cell stage in comparison to bone marrow derived cells.

Materials and Experimental Methods

Osteogenesis

Osteogenesis was carried out according to Chemicon osteogenesis kit (cat no. scr028, Millipore, MA, USA)

Osteogenesis induction medium

Osteogenesis induction medium was freshly made prior to each medium exchange using the kit components (See Table 3, below).

Table 3: Osteogenesis medium components

Component	Stock concentration	Amount	Final con
DMEM low glucose (Invitrogen, Gibco)		8.7 ml	87 %
Serum (heat inactivated)		1 ml	10 %
dexamethasone	1 mM	1 µl	0.1 µM
Asorbic Acid-2-Phosphate solution	0.1 M	20 µl	0.2 mM
Glycerol-2-Phosphate Solution	1 M	100 µL	10 Mm
L-glutamine	X 100	100 µl	X 1
Pen & Strep	X 100	100 µl	X 1

To arrive at 1 mM dexamethasone solution, 900 µl ethanol was added to 100 µl dexamethasone 10 mM solution. Stock solution was stored with the rest of the kit's components at -20 °C. A 50 ml serum vial was heat inactivated, divided into 5 ml aliquots and kept at -20 °C until use.

Coating 24-well tissue culture plates

A coating mixture comprising 12 µg/ml vitronectin and 12 µg/ml collagen (both included in the kit) was prepared by diluting each with 1 x PBS.

The coating mixture was then added to the wells to cover the well surfaces (5 wells x 2 plates were prepared). Plates were incubated overnight at room temperature. The coating mixture was then removed and the wells were rinsed once with PBS. Plates were aspirated right before use.

Cell Growth

Placenta derived cells (plc11-3-1) or bone marrow derived cells (BM108) were plated (200,000 cells per well) in 1 ml growth medium comprising DMEM (Invitrogen, Gibco), 10 % FCS (Invitrogen, Gibco), 2 Mm L-glutamine (Sigma-Aldrich), 45 µg/ml Gentamicin-IKA (Teva Medical) and 0.25 µg/ml Fungizone (Invitrogen, Gibco). Placenta derived cells (4 wells x 2 plates) or bone marrow derived cells (1 well x 2 plates) were grown until 100 % confluent (typically overnight) before initiating osteogenic differentiation.

When cells reached 100 % confluence, growth medium was aspirated and replaced with 1 ml osteogenesis induction medium (differentiation day 1). Osteogenesis induction medium was replaced with fresh medium every 2-3 days for a total of 14-17 days.

As a control, one of the two plates (for each of the cell types) was not incubated with osteogenesis differentiation medium but rather with the growth medium (described hereinabove).

On day 17, osteocytes were fixed and stained with Alizarin Red Solution as depicted in detail below.

Staining Protocol

Osteocyte staining was performed by first carefully aspirating the medium from each well (carefully as to not aspirate the cells). Cells were then fixed by incubating in iced cold 70 % ethanol for 1 hour at room temperature. The alcohol was then carefully aspirated and the cells were rinsed twice with water (5-10 minutes each wash). The water was then aspirated and alizarin red solution (500-1000 µl) was added to the cells. Cells were incubated with alizarin red solution at room temperature for 30 minutes. Alizarin red was removed and the cells were washed 4 times with 1 ml water and aspirated after each wash. Finally, 1-1.5 ml water was added to each well to prevent cell drying. The plates were microscopically visualized by an inverted Nikon microscope.

Experimental results

Osteocyte differentiation of placenta- or bone marrow- derived adherent cells in osteogenic induction medium resulted in differentiation of over 50 % of the bone marrow cells, as demonstrated by positive alizarin red staining (Figure 8B). On the contrary, none of the placental derived cells of the present invention showed any signs of osteogenic differentiation (see Figure 8E and Table 4, below).

Table 4: Differentiation summary

	BM 108+ BM109	PLC-11-3-1	PLC-8-2-1	Plc-15-3-4-2	Plc 4-3-1
Osteocytes	+++	-	-	-	-
Adipocytes	+++	-	-	-	-

EXAMPLE 5

Comparison of osteocyte differentiation of 2D adherent cells of the present invention and bone marrow cells in modified growth medium

The adherent cells of the present invention (from placenta origin, at their 2D adherent cell stage) or bone marrow derived cells were grown under osteocyte differentiation stimulating conditions in a modified osteogenic medium comprising Vitamin D and higher concentrations of dexamethasone.

Materials and Experimental Methods

Osteogenesis induction medium

Osteogenesis induction medium was freshly made prior to each medium exchange using the components listed in Table 5, below, along with Vitamin D.

Table 5: Osteogenesis medium components

Component	Stock con	Amount	Final con
DMEM high glucose (Biological Industries, Bet Haemek, Israel)		8.7 ml	87 %
L-glutamine	X 100	100 µl	X 1
Serum (heat inactivated)		1 ml	10 %
Dexamethasone (Chemicon)	10 mM	10 µl	10 µM
Asorbic Acid-2-Phosphate solution (Chemicon)	0.1 M	20 µl	0.2 mM
Glycerol-2-Phosphate Solution (Chemicon)	1 M	100 µL	10 Mm
Vitamin D (Sigma)	10 µM	10 µL	10 nM
Gentamycin (Biological Industries, Bet Haemek, Israel)	X 100	100 µl	X 1

A 50 ml serum vial was heat inactivated, divided into 5 ml aliquots and kept at -20 °C until use.

Coating 48-well tissue culture plates

A coating mixture comprising 12 µg/ml vitronectin and 12 µg/ml collagen (both from Chemicon) was prepared by diluting each with 1 x PBS.

The coating mixture was then added to the wells to cover the well surfaces (5 wells x 2 plates were prepared). Plates were incubated overnight at room temperature. The coating mixture was then removed and the wells were rinsed once with PBS. Plates were aspirated right before use.

Cell Growth

Placenta derived cells (PLC 8-2-1, PLC 15 3-4-2 or PLC 19-4-3-1 fetal cells) were plated (100,000 cells per well) in 0.5 ml growth medium comprising DMEM (Invitrogen, Gibco), 10 % FCS (Invitrogen, Gibco), 2 Mm L-glutamine (Sigma-Aldrich), 45 μg/ml Gentamicin-IKA (Teva Medical) and 0.25μg/ml Fungizone (Invitrogen, Gibco) (4 wells x 2 plates). Bone marrow derived cells (BM109) were plated (150,000 cells per well) in 0.5 ml growth medium (as described above) (1 well x 2 plates). Cells were grown until 100 % confluent (typically overnight) before initiating osteogenic differentiation.

When cells reached 100 % confluence, growth medium was aspirated and replaced with 0.5 ml osteogenesis induction medium (differentiation day 1). Osteogenesis induction medium was replaced with fresh medium every 2-3 days for a total of 26 days.

As a control, one of the two plates (for each of the cell types) was not incubated with osteogenesis differentiation medium but rather with the growth medium (described hereinabove).

On day 26, osteocytes were fixed and stained with Alizarin Red Solution as depicted in detail below.

Staining Protocol

Osteocyte staining was performed by first carefully aspirating the medium from each well (carefully as to not aspirate the cells). Cells were then fixed by incubating in iced cold 70 % ethanol for 1 hour at room temperature. The alcohol was then carefully aspirated and the cells were rinsed twice with water (5-10 minutes each wash). The water was then aspirated and alizarin red solution (500-1000 μl) was added to the cells. Cells were incubated with alizarin red solution at room temperature for 30 minutes. Alizarin red was removed and the cells were washed 4 times with 1 ml water and aspirated after each wash. Finally, 1-1.5 ml water was added to each well to prevent cell drying. The plates were microscopically visualized by an inverted Nikon microscope.

Experimental results

Osteogenic differentiation of placenta- or bone marrow- derived adherent cells was performed by modification of the protocol described in Example 4, hereinabove,

according to previous teachings [Parloni et al. (2008) Stem Cells 26(2): 300-11]. The main difference between the growth conditions presented in Example 4 and the results presented herein was the addition of vitamin D to the differentiation medium and the higher concentrations of dexamethasone. As evident from the results, over 50 % of the bone marrow cells underwent differentiation into osteocytes, as demonstrated by positive alizarin red staining (see Figure 9B). However, none of the placental derived cells of the present invention showed any signs of osteogenic differentiation (see Figure 9E and Table 4, hereinabove).

EXAMPLE 6

Comparison of adipocyte differentiation of 2D adherent cells of the present invention and bone marrow cells

The new adherent cells of the present invention (from placenta origin) were grown under adipocyte differentiation stimulating conditions at their 2D adherent cell stage in comparison to bone marrow derived cells.

Materials and Experimental Methods

Adipogenesis

Adipogenesis was carried out according to Chemicon adipogenesis kit (Chemicon adipogenesis kit, cat no. scr020, Millipore, MA, USA)

Adipogenesis induction medium

Adipogenesis induction or maintenance mediums were freshly prepared prior to every medium exchange using the components depicted in Tables 6 and 7, below.

Table 6: Adipogenesis induction medium components

Component	Stock con	Amount	Final con
DMEM low glucose (Biological Industries, Bet Haemek, Israel)		4.4 ml	90 %
Serum (heat inactivated)		0.5 ml	10 %
Dexamethasone (Sigma)	10 mM	0.5 µl	1 µM
IBMX (Sigma)	0.5 M	5 µl	0.5 mM
Insulin (Sigma)	10 mg/ml	5 µL	10 µg/ml
Indomethacin (Sigma)	10 mM	50 µl	100 µM
Pen & Strep	X 100	50 µl	X 1

Table 7: Adipogenesis maintenance medium components

Component	Stock con	Amount	Final con
DMEM low glucose		4.4 ml	90 %
Serum (heat inactivated)		0.5 ml	10 %
Insulin	10 mg/ml	5 µL	10 µg/ml
Pen & Strep	X 100	50 µl	X 1

Cell Growth

Placenta derived cells (plc11-3-1) or bone marrow derived cells (BM108) were plated (200,000 cells per well) in 1 ml growth medium comprising DMEM (Invitrogen, Gibco), 10 % FCS (Invitrogen, Gibco), 2 Mm L-glutamine (Sigma-Aldrich), 45 µg/ml Gentamicin-IKA (Teva Medical) and 0.25 µg/ml Fungizone (Invitrogen, Gibco). Placenta derived cells (4 wells x 2 plates) or bone marrow derived cells (1 well x 2 plates) were grown until 100 % confluent (typically overnight) before initiating adipogenesis differentiation.

When cells reached 100 % confluence, growth medium was aspirated and replaced with 1 ml adipogenesis induction medium (differentiation day 1). Adipogenesis induction medium was replaced with fresh medium every 2-3 days for a total of 25 days (as depicted in detail in Table 8, hereinbelow). Of note, monolayers of adipogenic cells were extremely fragile and could easily dislodged from plates, therefore, medium changes were performed with gentle medium changes to avoid disruption of the lipid droplets.

As a control, one of the two plates (for each of the cell types) was not incubated with adipogenesis differentiation medium but rather with the growth medium (described hereinabove).

Table 8: Adipogenesis differentiation schedule

Day	Medium
1	Adipogenesis *Induction* medium
3	Adipogenesis *Induction* medium
5	Adipogenesis *Induction* medium
7	Adipogenesis *Maintenance* medium
9	Adipogenesis *Induction* medium
11	Adipogenesis *Induction* medium
13	Adipogenesis *Induction* medium
15	Adipogenesis *Maintenance* medium
17	Adipogenesis *Induction* medium
19	Adipogenesis *Induction* medium
21	Adipogenesis *Induction* medium

On day 25, adipocytes were fixed and stained with oil red solution as depicted in detail below.

Staining Protocol

Adipocyte staining was performed by first carefully aspirating the medium from each well (carefully as to not aspirate the cells). Cells were then fixed by incubating in 4 % Para formaldehyde for 30-40 minutes at room temperature. The fixative was then carefully aspirated and the cells were rinsed three times with PBS (5-10 minutes each wash). Next, the PBS was aspirated and the cells were rinsed twice in water. The water was then aspirated and oil red solution (500-1000 µl) was added to the cells. Cells were incubated with oil red solution at room temperature for 50 minutes. Oil red solution was removed and the cells were washed 4 times with 1 ml water and aspirated after each wash. Finally, 1-1.5 ml water was added to each well to prevent cell drying. The plates were microscopically visualized by an inverted Nikon microscope.

Preparation of oil red solution

Stock of 0.25 g oil red (Sigma) was used which was dissolved in 50 ml iso-propanol by incubating10-15 min in 37 °C bath.

For use, 30 ml of the stock stain was mixed with 20 ml DDW (left to stand for 10 minutes and then filtered with coffee filter paper). The oil red solution was prepared fresh for each use.

Experimental results

Adipocyte differentiation of placenta- or bone marrow- derived adherent cells in adipocyte induction medium resulted in differentiation of over 50 % of the bone marrow derived cells (see Figure 8C), as demonstrated by positive oil red staining and by typical morphological changes (e.g. accumulation of oil droplets in the cytoplasm). In contrast, none of the placental derived cells of the present invention differentiated into adipocytes (see Figure 8F and Table 4, hereinabove).

EXAMPLE 7

Comparison of adipocyte differentiation of 2D adherent cells of the present invention and bone marrow cells in modified growth medium

The adherent cells of the present invention (from placenta origin, at their 2D adherent cell stage) or bone marrow cells were stimulated to differentiate into adipocytes in a modified adipocyte medium comprising a higher level of Indomethacine.

Materials and Experimental Methods

Adipogenesis induction medium

Adipogenesis induction medium was freshly prepared prior to every medium exchange using the components depicted in Table 9, below.

Table 9: Adipogenesis induction medium components

Component	Stock con	Amount	Final con
DMEM low glucose		4.4 ml	90 %
Serum (heat inactivated)		0.5 ml	10 %
Dexamethasone (Sigma)	1 mM	5 µl	1 µM
IBMX (Sigma)	0.5 M	5 µl	0.5 mM
Insulin (Sigma)	10 mg/ml	5 µL	10 µg/ml
Indomethacin (Sigma)	10 mM	200 µl	100 µM
Gentamycine (Biological Industries)		10 µl	

Cell Growth

Placenta derived cells (PLC 8-2-1, PLC 15 3-4-2 or PLC 19-4-3-1 fetal cells) were plated (100,000 cells per well) in 0.5 ml growth medium comprising DMEM (Invitrogen, Gibco), 10 % FCS (Invitrogen, Gibco), 2 Mm L-glutamine (Sigma-Aldrich), 45 µg/ml Gentamicin-IKA (Teva Medical) and 0.25 µg/ml Fungizone (Invitrogen, Gibco) (5 wells x 2 plates).

Bone marrow derived cells (BM109) were plated (100,000 cells per well) in 0.5 ml growth medium comprising DMEM (Invitrogen, Gibco), 10 % FCS (Invitrogen, Gibco), 2 Mm L-glutamine (Sigma-Aldrich), 45 µg/ml Gentamicin-IKA (Teva Medical) and 0.25 µg/ml Fungizone (Invitrogen, Gibco) (4 well x 2 plates). Cells were grown until 100 % confluent (typically overnight) before initiating adipogenesis differentiation.

When cells reached 100 % confluence, growth medium was aspirated and replaced with 0.5 ml adipogenesis induction medium (differentiation day 1).

Adipogenesis induction medium was replaced with fresh medium every 2-3 days for a total of 3-4 weeks.

As a control, one of the two plates (for each of the cell types) was not incubated with adipogenesis differentiation medium but rather with the growth medium (described hereinabove).

On day 26, adipocytes were fixed and stained with oil red solution as depicted in detail below.

Staining Protocol

Adipocyte staining was performed by first carefully aspirating the medium from each well (carefully as to not aspirate the cells). Cells were then fixed by incubating in 4 % Para formaldehyde for 30-40 minutes at room temperature. The fixative was then carefully aspirated and the cells were rinsed three times with PBS (5-10 minutes each wash). Next, the PBS was aspirated and the cells were rinsed twice in water. The water was then aspirated and oil red solution (500-1000 μl) was added to the cells. Cells were incubated with oil red solution at room temperature for 50 minutes. Oil red solution was removed and the cells were washed 3 times with 1 ml double distilled water and aspirated after each wash. Finally, 1-1.5 ml water was added to each well to prevent cell drying. The plates were microscopically visualized by an inverted Nikon microscope.

Preparation of oil red solution

Stock of 0.25 g oil red (Sigma) was used which was dissolved in 50 ml iso-propanol by incubating 10-15 min in 37 °C bath.

For use, 30 ml of the stock stain was mixed with 20 ml DDW (left to stand for 10 minutes and then filtered with coffee filter paper). The oil red solution was prepared fresh for each use.

Experimental results

Adipocyte differentiation of placenta- or bone marrow- derived adherent cells was performed by modification of the protocol in Example 6, hereinabove, according to previous teachings [Parloni et al. (2007), supra]. The main difference between the growth conditions presented in Example 6 and the results presented herein was the higher concentration of Indomethacine. As evident from the results, over 50 % of the bone marrow derived cells underwent differentiation into adipocytes (see Figure 9C), as

demonstrated by positive oil red staining and by typical morphological changes (e.g. accumulation of oil droplets in the cytoplasm). In contrast, none of the placental derived cells of the present invention exhibited morphological changes typical of adipocytes (see Figure 9F and Table 4, hereinabove).

EXAMPLE 8

Biodistribution of PLX-C

Materials and Experimental Methods

Transfection of PLX-C cells with Luciferase expression vector

PLX-C cells were stably infected with a lentiviral construct expressing the luciferase gene under the CMV promoter (Figure 10).

Production of infecting virus

293TN producer cells were grown in DMEM medium (Gibco) supplemented with serum and antibiotics for 2-3 days (50-70 % confluency) prior to transfection. A mixture of 10 µg of the packaging plasmid and 2 µg of expression construct and 20 µl of Plus™ Reagent (Invitrogen) were added to 400 µl of DMEM without supplements. The mixture was incubated for 15 min at room temperature (RT) and Lipofectamine™ (30 µl dilutes in 400 µl of DMEM were added). The mixture was incubated at RT for 15 min. 293TN cells were washed and transferred to 2 % serum media and transfection mixture was added. Cells were incubated in CO2 incubator at 37 °C over night and medium was collected 24 - 60 hrs post infection. Peak virus production was achieved after 48 hrs. Medium was collected, and centrifuged at 3000 rpm at room temperature for 5 minutes to pellet cell debris. Following centrifugation, the supernatant was filtered through Millex-HV 0.45 µm PVDF filters (Millipore, Cat. #SLHVR25LS).

Infection of PLX-C

PLX-C cells were seeded in a 24-well plate at a density of 0.6-1 x 10^5 cells per well in complete medium 24 hours prior to viral infection. After 24 hrs, 0.5 ml of virus suspension (diluted in complete medium with Polybrene at a final concentration of 5-8 µg/ml) was added. Cells were incubated for 24 hrs, then medium was replaced by complete DMEM medium and cells were incubated at 37 °C with 5 % CO2 overnight. At day 4, the culture reached confluency and was split by 1:3 to 1:5, cells were allowed

to grow for 48 hours in complete DMEM then cells were analyzed for Luciferase expression.

Efficiency rates of infection were close to 100 %. Evaluation of luminescence in living cells and in living mice was performed using the IVIS Lumina Imaging system, which included a highly sensitive CCD camera that captured the luciferase luminescence signal.

Two weeks post infection 2 x 10^6 cells were injected IM or IV into SCID/Beige, NOD/SCID, SCID and Balb/C mice. Injected cells were monitored using the described IVIS system.

Experimental results

As evident from the results, PLX-C cells continued to divide following infection, and expression levels of Luciferase in the growing cells remained strong and stable (Figure 11).

Once PLX-C cells were injected into Balb/C mice, the biodistribution pattern was examined. As evident from the results, cells disappeared 72 hrs post IM injection (data not shown). However, PLX-C cells retained constant high levels of Luciferase expression, in vitro, for over three weeks (data not shown).

As shown in Figures 12A-D, cells injected IM into SCID/Beige mice immunodeficient mice retained up to 5 days at the site of injection and were not observed thereafter. PLX-C cells injected IV into SCID/Beige mice migrated after 24 hrs to the lungs, then to the site of injection (presumably homing to site of injury). Afterwards cells disappear gradually and were not observed after 3-4 weeks.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission

that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

Claims

1. A method of culturing adherent cells from a placenta tissue, the method comprising culturing the adherent cells from the placenta tissue under 3 dimensional (3D) culturing conditions which allow cell expansion, said conditions comprising
perfusion, wherein said perfusion is adjusted according to the glucose concentration of the culture medium,

and

wherein said 3D culturing conditions

(i) comprise a 3D bioreactor;

(ii) comprise an adherent material selected from the group consisting of glass, plastic, polyester, polypropylene, polystyrene, dextran and collagen; and

(iii) are effected for at least 3 days; and

wherein culturing of said cells is effected until at least 10% of said cells are proliferating.

2. The method of claim 1, wherein said culture medium is changed at said glucose concentration of about 550 mg/L.

3. The method of claims 1 or 2, wherein said 3D culturing conditions are effected for 4-12 days.

ADHERENT CELLS FROM PLACENTA TISSUE AND USE THEREOF IN THERAPY

ABSTRACT

A method of culturing adherent cells from a placenta or adipose tissue is disclosed. The method comprising culturing the adherent cells from the placenta or adipose tissue under 3 dimensional (3D) culturing conditions which allow cell expansion, the conditions comprising perfusion.

No suitable figure