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(54) Title: ADHERENT CELLS FROM ADIPOSE OR PLACENTA TISSUES AND USE THEREOF IN THERAPY

(57) Abstract: A method of treating ischemia in a subject in need thereof is disclosed. The method comprising administering to
the subject a therapeutically effective amount of adherent cells of a tissue selected from the group consisting of a placenta and an
adipose tissue, thereby treating the ischemia in the subject. A method of treating a medical condition requiring connective tissue
regeneration and/or repair is also disclosed.
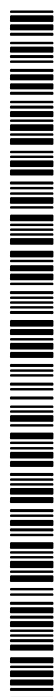
ADHERENT CELLS FROM ADIPOSE OR PLACENTA TISSUES AND USE THEREOF IN THERAPY

FIELD AND BACKGROUND OF THE INVENTION

The invention relates to methods of treating diseases using adherent cells from adipose or placenta tissues, more specifically, to methods of treating ischemia and/or medical conditions requiring connective tissue regeneration and/or repair using the adherent cells.

In the developing medical world a growing need exists for large amounts of adult stem cells for the purpose of cell engraftment and tissue engineering. In addition, adult stem cell therapy is continuously developing for treating and curing various conditions such as hematopoietic disorders, heart disease, Parkinson's disease, Alzheimer's disease, stroke, burns, muscular dystrophy, autoimmune disorders, diabetes and arthritis.

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 mesenchymal mature 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].

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. 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].

The present inventors have previously devised three dimensional (3D) culturing conditions suitable for expansion of placental derived MSCs (PCT Application No. IL2007/000380) fully incorporated herein by reference in its entirety.

Leading clinical uses of MSCs are summarized infra.

Ischemia

Peripheral arterial disease (PAD)

Peripheral arterial disease (PAD) is a chronic disease that progressively restricts blood flow in the limbs that can lead to serious medical complications. This disease is often associated with other clinical conditions, including hypertension, cardiovascular disease, hyperlipidemia, diabetes, obesity and stroke. Critical Limb Ischemia (CLI) is used to describe patients with chronic ischemia induced pain, ulcers, tissue loss or gangrene in the limb. CLI represents the end stage of PAD patients who need comprehensive treatment by a vascular surgery or vascular specialist. In contrast to coronary and cerebral artery disease, peripheral arterial disease (PAD) remains an under-appreciated condition that despite being serious and extremely prevalent is rarely diagnosed and even less frequently treated. Consequently, CLI often leads to amputation or death and mortality rates in PAD patients exceed that of patients with myocardial infarction and stroke.

In attempts to treat ischemic conditions, various adult stem cells have been used. Thus, co-culturing of adipose tissue derived stromal cells (ADSC) and endothelial cells (EC) resulted in a significant increase in EC viability, migration and tube formation mainly through secretion of VEGF and HGF. Four weeks after transplantation of the stromal cells into the ischemic mouse hind limb the angiogenic scores were improved [Nakagami et al., J Atheroscler Thromb (2006) 13(2): 77-81]. Moon et al. [Cell Physiol

Biochem. (2006) 17: 279-90] have tested the ability of adipose tissue-derived progenitor cells (ADSC) to treat limb ischemia in immunodeficient mice and demonstrated a significant increase in the laser Doppler perfusion index in ADSC-transplanted group.

In addition, when umbilical cord blood (UCB)-derived mesenchymal stem cells were transplanted into four men with Buerger's disease who had already received medical treatment and surgical therapies, ischemic rest pain, suddenly disappeared from their affected extremities [Kim et al., Stem Cells (2006) 24(6): 1620-6]. Moreover, transplantation of human mesenchymal stem cells isolated from fetal membranes of term placenta (FMhMSC) into infarcted rat hearts was associated with increased capillary density, normalization of left ventricular function, and significant decrease in scar tissue, which was enhanced when the stem cells were preconditioned with a mixed ester of hyaluronan with butyric and retinoic acid [Ventura et al., (2007) J. Biol. Chem., 282: 14243-52].

Stroke

Stroke is one of the leading causes of death around the world, causing approximately 9 % of all deaths and consuming about 2–4 % of total health-care costs. Although there has been a constant reduction in stroke mortality in developed countries, probably due improved control of stroke risk factors (especially high blood pressure, diabetes and cigarette smoking), stroke still leads to permanent damage (e.g. tissue damage, neurological damage).

New treatment regimens for stroke include stem cell therapy. Transplantation of stem cells or progenitors into the injured site, either locally or via intravenous routes, to replace nonfunctional cells, enhance proliferation and/or differentiation of endogenous stem or progenitor cells and supply necessary immune modulators has been contemplated and stand as the major cell-based strategy. Potential sources of stem/progenitor cells for stroke include fetal neural stem cells, embryonic stem cells, neuroteratocarcinoma cells, umbilical cord blood-derived non-hematopoietic stem cells, bone marrow-derived stem cells and placental-derived mesenchymal stem cells [Andres et al., Neurosurg Focus (2008) 24(3-4): E16].

In a recent study, Koh et. al. [Koh et al., Brain Res. (2008)] examined the neuroprotective effects and mechanisms of implanted human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) in an ischemic stroke rat model. Twenty days

after the induction of in-vitro neuronal differentiation, hUC-MSCs displayed morphological features of neurons and expressed neuronal cell markers and neuronal factors (e.g. glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor). Furthermore, in-vivo implantation of the hUC-MSCs into the damaged

5   hemisphere of immunosuppressed ischemic stroke rats improved neurobehavioral function and reduced infarct volume relative to control rats. Three weeks after implantation, hUC-MSCs were present in the damaged hemisphere and expressed neuron-specific markers, yet these cells did not become functionally active neuronal cells.

10   ***Orthopedic applications***

Various conditions and pathologies require connective tissue (e.g., bone, tendon and ligament) regeneration and/or repair. These include, for example, bone fractures, burns, burn wound, deep wound, degenerated bone, various cancers associated with connective tissue loss (e.g., bone cancer, osteosarcoma, bone metastases), and articular

15   cartilage defect.

The use of autologous BM-MSCs to enhance bone healing has been described for veterinary and human orthopedic applications and include percutaneous injection of bone marrow for ligament healing (Carstanjen et al., 2006), treatment of bone defects by autografts or allografts of bone marrow in orthopedic clinic (Horwitz et al., 1999,

20   Horwitz et al., 2002), regeneration of critical-sized bone defect in dogs using allogeneic [Arinzeh TL, et al., J Bone Joint Surg Am. 2003, 85-A(10):1927-35] or autologous [Bruder SP, et al., J Bone Joint Surg Am. 1998 Jul;80(7):985-96] bone marrow-MSCs loaded onto ceramic cylinder consisting of hydroxyapatite-tricalcium phosphate, or in rabbit using allogeneic peripheral blood derived MSCs (Chao et al., 2006.), and

25   extensive bone formation using MSCs implantation in baboon (Livingston et al, 2003).

Within the equine orthopedic field, mesenchymal stem cells of BM and adipose sources have been used experimentally for surgical treatment of subchondral- bone cysts, bone fracture repair [Kraus and Kirker-Head,Vet Surg (2006) 35(3): 232-42] and cartilage repair [Brehm et al., Osteoarthritis Cartilage (2006) 14(12): 1214-26; Wilke et

30   al., J Orthop Res (2007) 25(7): 913-25] and clinically in the treatment of overstrain induced injuries of tendons in horses. Furthermore, different therapeutic approaches have been used to promote suspensory ligament healing in horses (Herthel, 2001).

Herthel (2001) have demonstrated a novel biological approach to facilitate suspensory ligament healing that involves the intra lesional injection of autologous stem cells and associated bone marrow components to stimulate natural ligament regeneration.

Rabbit models for injured tendons showed that MSC-treated tissues were stronger and stiffer than natural repaired tissues (Gordon et al., 2005). In addition, seeding of cultured MSCs into a tendon gap resulted in significantly improved repair biomechanics (Young et al., 1998, Osiris Therapeutics, www.osiris.com).

Osiris Chondrogen (adult Mesenchymal Stem Cells) is being tested in patients in order to evaluate safety and efficacy. In MSC treated animals, surgically removed meniscal tissue was regenerated, the cartilage surface was protected, and lessened joint damage was observed in comparison to control animals. These benefits persisted in animal models at least through one year (Osiris Therapeutics, www.osiris.com).

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of treating ischemia in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of adherent cells of a tissue selected from the group consisting of a placenta and an adipose tissue, thereby treating the ischemia in the subject.

According to an aspect of some embodiments of the present invention there is provided a method of treating a medical condition requiring connective tissue regeneration and/or repair in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of adherent cells of a tissue selected from the group consisting of a placenta and an adipose tissue, thereby treating the medical condition requiring connective tissue regeneration and/or repair in the subject.

According to an aspect of some embodiments of the present invention there is provided a use of adherent cells from a tissue selected from the group consisting of a placenta and an adipose tissue for the manufacture of a medicament identified for treating ischemia.

According to an aspect of some embodiments of the present invention there is provided a use of adherent cells from a tissue selected from the group consisting of a

placenta and an adipose tissue for the manufacture of a medicament identified for treating a medical condition requiring connective tissue regeneration and/or repair.

According to an aspect of some embodiments of the present invention there is provided an article of manufacture comprising a packaging material which comprises a label for use in treating ischemia, the packaging material packaging a pharmaceutically effective amount of adherent cells of a tissue selected from the group consisting of a placenta and an adipose tissue.

According to an aspect of some embodiments of the present invention there is provided an article of manufacture comprising a packaging material which comprises a label for use in treating a medical condition requiring connective tissue regeneration and/or repair, the packaging material packaging a pharmaceutically effective amount of adherent cells of a tissue selected from the group consisting of a placenta and an adipose tissue.

According to some embodiments of the invention, the adherent cells are capable of suppressing immune reaction in the subject.

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

According to some embodiments of the invention, the ischemia is peripheral arterial disease (PAD).

According to some embodiments of the invention, the peripheral arterial disease (PAD) is critical limb ischemia (CLI).

According to some embodiments of the invention, the ischemia comprises ischemia of the central nervous system (CNS).

According to some embodiments of the invention, the ischemia is selected from the group consisting of peripheral arterial disease, ischemic vascular disease, ischemic heart disease, ischemic brain disease, ischemic renal disease and ischemic placenta.

According to some embodiments of the invention, the adherent cells are obtained from a three-dimensional (3D) culture.

According to some embodiments of the invention, the three-dimensional (3D) culture comprises a 3D bioreactor.

According to some embodiments of the invention, the culturing of the cells in the 3D culture is effected under perfusion.

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According to some embodiments of the invention, the culturing conditions of the three-dimensional culture comprise an adherent material selected from the group consisting of a polyester and a polypropylene.

According to some embodiments of the invention, the culturing of the cells is effected for at least 3 days.

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

According to some embodiments of the invention, the adherent cells comprise a positive marker expression selected from the group consisting of CD73, CD90, CD29 and CD105.

According to some embodiments of the invention, the adherent cells comprise a negative marker expression selected from the group consisting of CD3, CD4, CD45, CD80, HLA-DR, CD11b, CD14, CD19, CD34 and CD79.

According to some embodiments of the invention, the adherent cells comprise an expression profile essentially as described herein.

According to some embodiments of the invention, the adherent cells comprise cells comprising a stromal stem cell phenotype.

According to some embodiments of the invention, the stromal stem cell phenotype comprises T cell suppression activity.

According to some embodiments of the invention, the connective tissue comprises tendon, bone and/or ligament.

According to some embodiments of the invention, the medical condition requiring connective tissue regeneration and/or repair is selected from the group consisting of bone fracture, bone cancer, burn wound, articular cartilage defect and deep wound.

According to some embodiments of the invention, the medical condition is selected from the group consisting of a subchondral-bone cyst, a bone fracture, an osteoporosis, an osteoarthritis, a degenerated bone, a bone cancer, a cartilage damage, an articular cartilage defect, a degenerative disc disease, an osteogenesis imperfecta (OI), a burn, a burn wound, a deep wound, a delayed wound-healing, an injured tendon and an injured ligament.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, suitable methods and 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 not intended to be limiting.

Definitions of the specific embodiments of the invention as claimed herein follow.

According to a first embodiment of the invention, there is provided a method of treating a condition in a subject in need thereof, said method comprising the step of administering to the subject a therapeutically effective amount of adherent placental cells, wherein said condition is selected from the group consisting of ischemia, a condition requiring connective tissue regeneration and a condition requiring connective tissue repair.

According to a second embodiment of the invention, there is provided use of an article of manufacture comprising a packaging material, which comprises a label, for treating a condition in a subject in need thereof, wherein said condition is selected from the group consisting of ischemia, a condition requiring connective tissue regeneration and a condition requiring connective tissue repair, said packaging material packaging a pharmaceutically effective amount of adherent placental cells.

According to a third embodiment of the invention, there is provided adherent placental cells when used for treating a condition in a subject, wherein said condition is selected from the group consisting of ischemia, a condition requiring connective tissue regeneration and a condition requiring connective tissue repair.

According to a fourth embodiment of the invention, there is provided an article of manufacture comprising a packaging material, which comprises a label, when used for treating a condition in a subject, wherein said condition is selected from the group consisting of ischemia, a condition requiring connective tissue regeneration and a condition requiring connective tissue repair, said packaging material packaging a pharmaceutically effective amount of adherent placental cells.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is 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

the embodiments of the invention only, and are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIGs. 1A-G depict the bone-like microenvironment created in the bioreactor system containing 3-D carriers. Figures 1A-B are electron micrographs depicting the comparison of natural bone (Figure 1A) and the structure of the PluriX™ 3D carrier 7 days after seeding adherent cells, imitating the bone micro-environment (Figure 1B). Figures 1C-F are electron micrographs depicting the PluriX™ 3D matrix seeded with adherent cell, produced from bone marrow, 20 days (Figures 1C-D, magnified X 150 and 250 respectively) and 40 days (Figures 1E-F, magnified X 350 and 500 respectively) after seeding. Figure 1G is a diagram of the Plurix 3D plug flow bioreactor with separate parts defined by numbers: Culture medium reservoir (1), gas mixture supply (2), filter (3), injection point (4), column in which the 3D carriers are placed (5) flow monitor (6), flow valve (6a), separating container (7), cell growth analyzers (8); peristaltic pump (9), sampling point (10), dissolved O_2 measurement

electrode (11), pH measurement electrode (12), control system (13), fresh growth media (14), used growth media (15).

FIG. 2 is a graph depicting different production lots of adherent cells (Lots 5-8) originating from placenta, grown in 3D growth conditions within the bioreactor systems. Adherent cells (2 X 10^6) were seeded in the bioreactor at a density of 10000 - 15000 cells / a carrier. Following a 12 day culture 3D-adherent cells reached a density of between 150,000-250,000 cells /carrier or 22.5-37.5 X 10^6 in a bioreactor containing 150 carriers.

FIGs. 3A-B are bar graphs depicting difference in expression levels of expressed membrane markers in placenta derived 3D-adherent cell (dark purple) as compared to membrane markers in placenta cells cultured in conventional 2D culture conditions (light purple). Adherent cells were grown for 4-6 weeks in flasks (2D) or for 2-3 weeks in the bioreactor system, on polystyrene carriers (3D). Following harvesting from either flasks or carriers, cells were incubated and bound to a panel of monoclonal antibodies (MAb), which recognize membrane markers characteristic of adherent cells (Figure 3A), or hematopoietic cells (Figure 3B). Note the significantly higher expression of MSC membrane markers in 2D cultured cells as shown for CD90, CD105, CD73 and CD29 membrane markers, compared to MSC membrane markers expressed in 3D-cultured adherent cells, especially CD105 which showed 56 % expression in 3D cultured cells vs. 87 % in the 2D cultured cells (Figure 3A). Adherent cells of both 2D and 3D cultures, did not express any hematopoietic membrane markers (Figure 3B).

FIGs. 4A-D are bar graphs depicting a comparison of protein levels in adherent cells produced from the placenta cultured under 2D and 3D Conditions or conditioned media of same. Figures 4A-C depict levels of Flt-3 ligand (Figure 4A), IL-6 (Figure 4B) and SCF (Figure 4C) in pg/ml, normalized for 1 X 10^6 cells/ml, as analyzed by ELISA, in the conditioned media of 2D and 3D cultured adherent cells. Results represent one of three independent experiments. Figure 4D shows the expression levels of different cellular proteins, as analyzed by mass spectrometry with iTRAQ reagents labeled protein samples compared therebetween. Protein samples were taken from adherent cells grown under 2D (white bars) and 3D (grey bars) conditions. The figure represents one of two replica experiments. Note the difference in expression level of some of the proteins in cells and conditioned media of 2D and 3D culture conditions.

FIGs. 5A-D are micrographs depicting *in vitro* differentiation capability of placenta derived 3D-adherent cell to osteoblasts. Human placenta derived adherent cell were cultured in an osteogenic induction medium (DMEM containing 10 % FCS, 100 nM dexamethasone, 0.05 mM ascorbic acid 2-phosphate, 10 mM B-glycerophosphate)
5   for a period of 3 weeks. Figures 5A-B show cells expressing calcified matrix, as indicated by Alizzarin Red S staining. Figures 5C-D show control cells, which were not treated with osteogenic induction medium and maintained a fibroblast like phenotype and demonstrating no mineralization.

FIG. 6 is a graph depicting percentage of human CD45+ cells detected in bone
10   marrow (BM) of NOD-SCID mice, treated with chemotherapy (25 mg/kg busulfan intraperitoneal injections for two consecutive weeks) 3.5 weeks following transplantation. CD34+ cells (100,000) purified from mononuclear cord blood derived cells, were transplanted alone (5 mice, a) or co-transplanted with 0.5 X 10^6 placenta derived adherent cells cultured in 2D conditions (2D-adherent cell; 2 mice, b), or
15   placenta derived adherent cells cultured in 3D conditions (3D-adherent cell), in the pluriX^{TM} bioreactor (5 mice, c). BM was then collected from mice femurs and tibias. Human cells in the BM were detected by flow cytometry. The percentage of CD45 expressing human cells was determined by incubating cells with anti-human CD45-FITC. Note the higher percentage of human cells (hCD45+) in the bone marrow of mice
20   co-transplanted with 2D-adherent cell (b) as well as with 3D-adherent cell (c) in comparison to the percentage of human cells in the mice treated with HSCs alone (a). The higher engraftment seen in mice treated with 3D-adherent cell cultured cells in comparison to mice treated with 2D-adherent cell cultured cells indicates a higher therapeutic advantage unique to 3D cultured adherent cells.

25   FIGs. 7A-B are FACS analyses of human graft CD45+ cells in mice transplanted with CD34+ cells only (Figure 7A) in comparison to CD34+ cells together with adipose tissue derived adherent cells (Figure 7B). Note the significantly higher percentage of human hematopoietic population (hCD45+) (7A – 29 %) in a mouse co-transplanted with adipose tissue derived adherent cell in comparison to a mouse treated with human
30   CD34+ alone (7B -12 %).

FIG. 8A is a bar graph depicting a mixed lymphocyte reaction conducted between human cord blood mononuclear cells (CB), and equal amounts of irradiated

(3000 Rad) cord blood cells (iCB), human peripheral blood derived monocytes (PBMC), 2D cultured (2D) or 3D cultured (3D) placental derived adherent cells, or a combination of PBMC and 2D and 3D cultured placental derived adherent cells (PBMC+2D and PBMC+3D). Size of CB cell population is represented by the ^{3}H-thymidine uptake (measured in CPM) which was measured during the last 18 hours of culturing. Elevation in stimulated CB cell proliferation indicates an immune response of a higher level. Note the lower level of immune response exhibited by cells incubated with adherent cells, and, in particular, the reduction of CB immune response to PBMCs when co-incubated with adherent cells. Three replicates were made of each reaction.

FIG. 8B is a flow chart depicting production of 3D adherent cell from placentas by Celligen™ (designated PLX-C cells).

FIG. 8C is a diagram of a Celligen™ bioreactor vessel and ports adapted from The New Brunswick Scientific web site.

FIGs. 9A-B depict cell cycle analysis of 3D adherent cells manufacture by Plurix (designated PLX, Figure 9B) and by Celligen (designated PLX-C, Figure 9A). Cells were fixed in 70 % EtOH O.N, centrifuged and re-suspended in a Propidium Iodide (PI) solution and then analyzed by FACS.

FIGs. 10A-C depict expression of fibroblast-typical markers but not expression of endothelial typical markers on PLX-C. Figure 10A depicts negative expression of the endothelial marker CD31; Figure 10B depicts negative expression of the endothelial marker KDR; and Figure 10C depicts positive expression of the human fibroblast marker (D7-FIB). Of note, the red histograms for Isotype IgG1 (FITC) represent the negative control while the blue histograms represents the positively stained cells.

FIGs. 11A-D depict expression of stimulatory and co-stimulatory molecules on PLX-C cells. Figure 11A depicts PLX-C expression of CD80; Figure 11B depicts PLX-C expression of CD86; Figure 11C depicts PLX-C expression of CD40; and Figure 11D 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. 12A-B depict inhibition of lymphocyte proliferation by PLX-C. Figure 12A depicts MLR tests performed with 2×10^5 peripheral blood (PB) derived 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 12B 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. 13A-C depict PLX-C regulation of pro-inflammatory and anti-inflammatory cytokine secretion following co-culture with peripheral blood cells. Figures 13A-B depict secretion of IFNγ (Figure 13A) and TNFα (Figure 13B) following co-culture of human derived MNCs (isolated from peripheral blood) stimulated with ConA with PLX-C; Figure 13C 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.

FIG. 14 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. 15 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. 16A-D depict injection of 2×10^6 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 16A), day 4 (Figure 16B), day 6 (Figure 16C) and day 22 (Figure 16D) are presented.

FIG. 17 is a graph depicting increased perfusion in hip and foot of mice treated with the adherent cells of the invention (designated PLX-C). The figure depicts the median of the percent of perfusion in the mouse hip and foot. Blood flows on hip and foot was measured using a non contact laser Doppler from both sides on days 0, 6, 9, 14

and 21 post operation (shown are measurements on day 21). Results are expressed as the ratio of the blood flow in the ischemic limb to that in the normal limb during the experiment.

FIG. 18 is a graph depicting the *in vivo* assessment of limb function and ischemic damage. Semiquantitative assessment of impaired use of the ischemic limb was performed serially using the following score system: 3 = dragging of foot, 2 = no dragging but no plantar flexion, 1 = plantar flexion, and 0 = flexing the toes to resist gentle traction of the tail.

FIGs. 19A-C depict increased capillary density after PLX-C treatment. Figure 19A depicts capillary density in mice treated with PBS; Figure 19B depicts capillary density in mice treated with PLX-C cells; Figure 19C is a bar graph depicting the number of capillaries per muscle cells. Of note, increase capillary density was noted in PLX-C treated mice but not in control mice, following induced limb ischemia demonstrated by specific capillary staining.

FIGs. 20A-B depict reduced oxidative stress and endothelial inflammation following PLX-C administration. Figure 20A is a bar graph depicting oxidative stress (Nitrotyrosin staining); and Figure 20B is a bar graph depicting endothelial inflammation (VCAM evaluation). Of note, reduced oxidative stress and endothelial inflammation is noted in mice treated with PLX-C.

DESCRIPTION OF THE EMBODIMENTS OF THE INVENTION

The invention is of, in some embodiments, methods of increasing angiogenesis in a tissue and treating ischemia or medical conditions requiring connective tissue regeneration and/or repair using adherent cells of placenta or adipose tissues.

The principles and operation of the 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 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 invention to practice, the present inventors have uncovered that adherent cells from a placenta or an adipose tissue are highly efficient in increasing angiogenesis in a tissue and in treating ischemia and medical conditions requiring connective tissue regeneration and/or repair.

As is illustrated herein below and in Example 1-8 of the Examples section which follows, the present inventors were able to expand adipose and placenta-derived adherent cells which comprise stromal stem cells properties. Cells expanded accordingly were found viable, following cryo-preservation, as evidenced by adherence and re-population assays (see Example 1). Flow cytometry analysis of placenta-derived adherent cells uncovered a distinct marker expression pattern (see Figures 3A-B). As is further shown in Example 6 of the Examples section which follows, implantation of placental derived adherent cells significantly induced blood flow in the hip and foot (Figure 17) of mice subjected to artery ligation (the ischemic hind limb model), significantly improved limb function (Figure 18), increased capillary density (Figures 19A-C) and reduced oxidative stress and endothelial inflammation (Figures 20A-B).

Thus, according to one aspect of the invention, there is provided a method of increasing angiogenesis in a tissue. The method is effected by contacting the tissue with adherent cells of a tissue selected from the group consisting of a placenta and an adipose tissue, thereby increasing the angiogenesis in the tissue.

As used herein the phrase "increasing angiogenesis in a tissue" refers to increasing (inducing, upregulating) the process of generating new capillary blood vessels in a tissue.

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 female 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.

Placenta or adipose tissue derived adherent cells can be propagated using two dimensional or three dimensional culturing conditions.

Conditions for propagating adherent cells in 2D culture are further described hereinbelow and in the examples section which follows.

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 while allowing the cells to grow in more than one layer. 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 an adipose or placental 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. The term "perfuse" or "perfusion" used herein refers to the act of pouring or passaging a fluid over or through an organ or tissue. 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 or inner parts of the placenta, see Example 1) and maternal (*i.e.*, decidua basalis, and

5   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.

10   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.

15   Isolated adherent cells from 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

20   between 20 microns to 1 mm. The cells are then subjected to differential centrifugation directly in media or over a Ficoll or Percoll or other particulate gradient. 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).

In addition to placenta or adipose tissue derived adherent cells, the invention

25   also envisages the use of adherent cells from other cell sources which are characterized by stromal stem cell phenotype (as will be further described herein below). Tissue sources from which adherent cells can be retrieved include, but are not limited to, cord blood, scalp, hair follicles [e.g. as described in Us Pat. App. 20060172304], testicles [e.g., as described in Guan K., et al., Nature. 2006 Apr 27;440(7088):1199-203], human

30   olfactory mucosa [e.g., as described in Marshall, CT., et al., Histol Histopathol. 2006 Jun;21(6):633-43], embryonic yolk sac [e.g., as described in Geijsen N, Nature. 2004 Jan 8;427(6970):148-54] and amniotic fluid [Pieternella et al. (2004) Stem Cells

22:1338-1345], all of which are known to include mesenchymal stem cells. Adherent cells from these tissue sources can be isolated by culturing the cells on an adherent surface, thus isolating adherent cells from other cells in the initial population.

Regardless of the origin (e.g., placenta or adipose tissue), cell retrieval is preferably effected under sterile 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. Culturing may proceed under 2D conditions as described in Example 4 of the Examples section and cells may be further transferred to 3D conditions

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, chondronectin, laminin), a collagen, a poly L lactic acid 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 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).

It will be appreciated that in case the adherent cells of the invention are administered to a human subject, the cells and the culture medium (e.g., with the above described medium additives) should be substantially xeno-free, i.e., devoid of any animal contaminants e.g., mycoplasma. For example, the culture medium can be supplemented with a serum-replacement, human serum and/or synthetic or recombinantly produced factors.

As mentioned, once adherent cells are at hand they may be passaged to two dimensional or three dimensional settings (see Examples 1 and 4 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 conditions (as mentioned hereinabove).

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
5   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, a CelliGen
10   Plus® bioreactor system (New Brunswick Scientific (NBS) or a BIOFLO 310 bioreactor system (New Brunswick Scientific (NBS).

As shown Example 4 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
15   cultures can be directly 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
20   limited to, a continuous stirred tank bioreactor, where a culture medium is continuously fed into the bioreactor and a product is continuously drawn out, to maintain a time-constant steady state within the reactor. A stirred tank bioreactor with a fibrous bed basket 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
25   of a central draught tube flowing up while forming bubbles, and disengaging exhaust gas at the top of the column], a cell seeding perfusion bioreactor with Polyactive foams [as described in Wendt, D. et al., Biotechnol Bioeng 84: 205-214, (2003)] tubular poly-L-lactic acid (PLLA) porous scaffolds in a Radial-flow perfusion bioreactor [as described in Kitagawa et al., Biotechnology and Bioengineering 93(5): 947-954 (2006).
30   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.

Cell seeding is preferably effected 100,000-1,500,000 cells / mm at seeding. In an exemplary embodiment a total of $150 \pm 30 \times 10^6$ cells are seeded, $3\text{-}5 \times 10^6$ cell / gr carrier are seeded, or $0.015\text{-}0.1 \times 10^6$ cell / ml are seeded.

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, 10 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.

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+, CD3-, CD4-, CD34-, CD45-, CD80-, CD19-, CD5-, CD20-, CD11B-, CD14-, CD19-, CD79-, HLA-DR-, 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 4 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 (don't stimulate T cells and conversely suppress same), hematopoietic stem cell support activity, as well as any of adipogenic, hepatogenic, osteogenic and neurogenic differentiation.

Any of these structural or functional features can be used to qualify the cells of the invention (see Examples 4 of the Examples section which follows).

Populations of cells generated according to the present teachings are characterized by a unique protein expression profile as is shown in Example 1 of the Examples section. Thus for example, adherent cells of placenta or adipose tissue generated according to the present teachings are capable of expressing and/or secreting high levels of selected factors. For example, such cells express or secrete SCF, Flt-3, H2A histone family (H2AF) or Aldehyde dehydrogenase X (ALDH X) at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or even 12 fold higher than that expressed or secreted by adherent cells of placenta or adipose tissue grown in a 2D culture. Additionally or alternatively, population of cells of the invention secrete or express IL-6, eukaryotic translation elongation factor 2 (EEEF2), reticulocalbin 3, EF-hand calcium binding domain (RCN2) or calponin 1 basic smooth muscle (CNN1) at a level least 2, 3 or 5 fold higher than that expressed or secreted by adherent cells of placenta or adipose tissue grown in a 2D culture. Additionally or alternatively, population of cells of the invention are characterized by lower level of expression of various other proteins as compared to 2D cultured cells. Thus for example, secrete or express less than 0.6, 0.5, 0.25 or 0.125 of the expression level of heterogeneous nuclear ribonucleoprotein H1 (Hnrph1), CD44 antigen isoform 2 precursor, 3 phosphoadenosine 5 phosphosulfate synthase 2 isoform a (Papss2) or ribosomal protein L7a (rpL7a) expressed or secreted by adherent cells of placenta or adipose tissue grown in a 2D culture.

As is shown in Examples 3-4 of the Examples section which follows, the adherent cells, and particularly 3D-adherent cells, were found to suppress the immune reaction of human cord blood 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.

According to one embodiment of the invention, the adherent cells of the invention are characterized by a higher immunosuppressive activity than that of adherent cells of the placenta or the adipose tissue grown in a two-dimensional (2D) culture.

According to one embodiment of the invention, the immunosuppressive activity comprises reduction in T cell proliferation.

As mentioned hereinabove and described in Example 6 of the Examples section which follows, the adherent cells of the invention induced angiogenesis *in vivo* (e.g., blood flow in the hip and leg), significantly improved limb function of animals subjected to arterial ligation, increased capillary density and reduced oxidative stress and endothelial inflammation. Furthermore, as described in detail in Example 7 of the Examples section which follows, the adherent cells of the invention significantly improved recovery from stroke in a rat model.

Thus, according to another aspect of the invention, there is provided a method of treating ischemia in a subject in need thereof. The method is effected by administering to the subject a therapeutically effective amount of the adherent cells of the invention, thereby treating the ischemia in the subject.

The term "ischemia" as used herein refers to any pathology (disease, condition, syndrome or disorder) characterized by or associated with insufficient angiogenesis. Examples include, but are not limited to, a peripheral arterial disease (PAD) such as limb ischemia and critical limb ischemia (CLI), ischemic heart disease, ischemic brain disease (e.g. stroke), delayed wound-healing, delayed ulcer healing, reproduction associated disorders, arteriosclerosis, ischemic vascular disease, 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, vascular disease of the kidney, peripheral arterial

disease, limb ischemia, lower extremity ischemia, 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 and ischemic placenta.

5   As used herein the term "treating" refers to inhibiting or arresting the development of a pathology (e.g., ischemia) 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,

10   remission or regression of a pathology. The term "treating" may also refer to alleviating or diminishing a symptom associated with the pathology.

As used herein the phrase "subject in need thereof" refers to any subject (e.g., mammal), such as a human subject who is diagnosed with or suffers from the pathology.

As mentioned hereinabove and described in Example 8 of the Examples section

15   which follows, the present inventors have found that the adherent cells of the invention are capable of connective tissue regeneration and/or repair.

Thus, according to yet an additional aspect of the invention, there is provided a method of treating a medical condition requiring connective tissue regeneration and/or repair in a subject in need thereof. The method is effected by administering to the

20   subject a therapeutically effective amount of the adherent cells of the invention.

The phrase "connective tissue" refers to a supporting framework tissue comprising strands of collagen, elastic fibers (e.g., between and around muscle and blood vessels) and simple cells. Examples of connective tissues include, but are not limited to dense connective tissue (e.g., ligament, tendon, periodontal ligament), areolar

25   connective tissue (e.g., with proteinaceous fibers such as collagen and elastin), reticular connective tissue, adipose tissue, blood, bone, cartilage, skin, intervertebral disc, dental pulp, dentin, gingival, extracellular matrix (ECM)-forming cells, loose connective tissue and smooth muscle cells.

As used herein the phrase "medical condition requiring connective tissue

30   regeneration and/or repair" refers to any pathology characterized by connective tissue damage (*i.e.*, non-functioning tissue, cancerous or pre-cancerous tissue, broken tissue, fractured tissue, fibrotic tissue, or ischemic tissue) or loss (e.g., following a trauma, an

infectious disease, a genetic disease, and the like). Non-limiting examples of such pathologies include, bone fracture, bone cancer (e.g., osteosarcoma, bone cancer metastasis), burn wound, articular cartilage defect and deep wound.

The phrase "administering to the subject" refers to the introduction of the cells

5   of the invention to target tissue. The cells can be derived from the recipient or from an allogeneic or xenogeneic donor. This phrase also encompasses "transplantation", "cell replacement" or "grafting" of the cells of the invention into the subject.

The subject may be any mammal in need of connective tissue regeneration and/or repair including e.g. human or domesticated animals including, but not limited to, horses

10   (i.e. equine), cattle, goat, sheep, pig, dog, cat, camel, alpaca, llama and yak.

According to an embodiment of the present teachings, the adherent cells of the present invention may be used to treat conditions including subchondral-bone cysts, bone fractures, osteoporosis, osteoarthritis, degenerated bone, various cancers associated with connective tissue loss (e.g., bone cancer, osteosarcoma, bone metastases), cartilage

15   damage, articular cartilage defect, degenerative disc disease, osteogenesis imperfecta (OI), burns, burn wounds, deep wounds, delayed wound-healing, injured ligaments and injured tendons e.g. overstrain-induced injuries of tendons in horses and other subjects in need thereof (as stated above).

Cells which may be administered in accordance with this aspect of the invention

20   include the above-described adherent cells which may be cultured in three-dimensional or two dimensional settings as well as mesenchymal and-non mesenchymal partially or terminally differentiated derivatives of same.

Methods of deriving lineage specific cells from the stromal stem cells of the invention are well known in the art. See for example, U.S. Pat. Nos. 5,486,359,

25   5,942,225, 5,736,396, 5,908,784 and 5,902,741.

The cells may be naïve or 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 (i.e., allogeneic or xenogeneic) of fresh or frozen (e.g., cryo-preserved) preparations.

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

Since non-autologous cells may induce an immune reaction when administered to the body several approaches have been developed to reduce the likelihood of rejection of non-autologous cells. These include either suppressing the recipient immune system or encapsulating the non-autologous cells in immunoisolating,

5   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).

10   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,

15   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.

20   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,

25   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.

30   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, TNF.alpha. 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.

In any of the methods described herein, 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 of a tissue selected from the group consisting of placenta and adipose tissue, which are obtained from a three-dimensional culture), 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.

According to a preferred embodiment of the invention, the pharmaceutical carrier is an aqueous solution of saline.

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.

One may administer the pharmaceutical composition in a systemic manner (as detailed hereinabove). Alternatively, one may administer the pharmaceutical composition locally, for example, via injection of the pharmaceutical composition directly into a tissue region of a patient.

Pharmaceutical compositions of the invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

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. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from *in vitro* and cell culture assays. Preferably, a dose is formulated in an animal model to achieve a desired concentration

or titer. Such information can be used to more accurately determine useful doses in humans.

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.

The data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition, (see e.g., Fingl, *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1). For example, Parkinson's patient can be monitored symptomatically for improved motor functions indicating positive response to treatment.

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, or physiological salt buffer.

Dosage amount and interval may be adjusted individually to levels of the active ingredient which are sufficient to effectively regulate the neurotransmitter synthesis by the implanted cells. Dosages necessary to achieve the desired effect will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the individual being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc. The dosage and timing of administration will be responsive to a careful and continuous monitoring of the individual changing condition. For example, a treated Parkinson's patient will be administered with an amount of cells which is sufficient to alleviate the symptoms of the disease, based on the monitoring indications.

Models for ligament injury include, but are not limited to, rabbit model of anterior cruciate ligament reconstruction using mesenchymal stem cells [Jit-Kheng et al., Arthroscopy (2004) 20(9): 899-910], goat model for use of long-term bioresorbable scaffolds for anterior cruciate ligament repair [Altman et al., J Am Acad Orthop Surg.

5   (2008) 16(4):177-187]. Models for tendon repair include, but are not limited to, adult New Zealand White rabbit model for autologous mesenchymal stem cell-mediated repair of tendon [Awad et al., Tissue Eng. (1999) 5(3):267-77]. Models for bone repair were described in e.g. Stem Cells in Endocrinology, Humana Press (2005) 183-206, describing the manipulation of mesenchymal stem cells for bone repair.

10   Following transplantation, the cells of the invention preferably survive in the diseased area for a period of time (e.g. about 1 month), such that a therapeutic effect is observed.

Compositions including the preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate

15   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

20   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

25   the U.S. Food and Drug Administration for prescription drugs or of an approved product insert.

The adherent cells of the invention can be suitably formulated as pharmaceutical compositions which can be suitably packaged as an article of manufacture. Such an article of manufacture comprises a packaging material which comprises a label for use

30   in increasing angiogenesis in a tissue, treating ischemia and/or treating a pathology requiring connective tissue regeneration and/or repair, wherein the packaging material packaging the adherent cells of the invention.

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

5 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, muscular diseases, nephric diseases, diseases related to reproduction, connective tissue diseases and systemic diseases.

10 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

15 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.

20 *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

25 (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

30 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 1 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.

5   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.*,

10   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

15   (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.

20   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 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

25   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,

30   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

5   (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

10   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)

15   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).

20   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).

25   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,

30   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 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 "about" refers to ± 10 %.

Additional objects, advantages, and novel features of the invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the invention as delineated hereinabove and as claimed in the claims section below finds 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 invention include molecular, biochemical, microbiological andrecombinant 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

5   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

10   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.

15   *EXAMPLE 1*

PRODUCTION AND CULTURING OF ADHERENT CELLS FROM BONE MARROW, PLACENTA AND ADIPOSE TISSUES

Adherent cells were cultured in a bioreactor system containing 3D carriers to produce 3D-adherent cells, characterized by a specific cell marker expression profile.

20   Growth efficiency was tested through cell count. The differentiation capacity of these cells was tested by culturing in a differentiation medium.

Materials and Experimental Procedures

Bone marrow adherent cells – Bone marrow (BM) adherent cells were obtained from aspirated sterna marrow of hematologically healthy donors undergoing open-heart

25   surgery or BM biopsy. Marrow aspirates were diluted 3-fold in Hank's Balanced Salts Solution (HBSS; GIBCO BRL/Invitrogen, Gaithersburg MD) and subjected to Ficoll-Hypaque (Robbins Scientific Corp. Sunnyvale, CA) density gradient centrifugation. Thereafter, marrow mononuclear cells (<1.077 gm/cm^3) were collected, washed 3 times in HBSS and resuspended in growth media [DMEM (Biological Industries, Beit

30   Ha'emek, Israel) supplemented with 10 % FCS (GIBCO BRL), 10^{-4} M mercaptoethanol (Merck, White House Station, NJ), Pen-Strep-Nystatin mixture (100 U/ml:100 µg/ml:1.25 un/ml; Beit Ha'Emek), 2 mM L-glutamine (Beit Ha'Emek)]. Cells from

individual donors were incubated separately in tissue culture flasks (Corning, Acton, MA) at 37 °C (5 % CO_2) with weekly change of culture media. Cells were split every 3-4 days using 0.25 % trypsin-EDTA (Beit Ha'Emek). Following 2-40 passages, when reaching 60-80 % confluence, cells were collected for analysis or for culturing in

5   bioreactors.

Placenta derived adherent cells - Inner parts of a full-term delivery placenta (Bnei Zion medical center, Haifa, Israel) were cut under sterile 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

10   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. Thereafter, cells were allowed to adhere to a plastic surface for 72 hours after which the media was changed every 3 - 4 days. When reaching 60-80 %

15   confluence (usually 10-12 days), cells were detached from the growth flask using 0.25 % trypsin-EDTA and seeded into new flasks. Cultured cells were thereafter collected for analysis or for culturing in bioreactors.

Adipose derived adherent cells – Adherent cells were obtained from human adipose tissue of liposuction procedures (Rambam Haifa, Israel). Adipose tissue was

20   washed extensively with equal volumes of PBS and digested at 37 °C for 30 minutes with collagenase (20 mg/ml). Cells were then washed with DMEM containing 10 % FCS, Pen-Strep-Nystatin mixture (100 U/ml:100 µg/ml:1.25 un/ml) and L- Glutamin and centrifuged at 1200 rpm for 10 minutes at room temperature (RT), resuspended with lysing solution (1:10; Biological Industries, Beit Ha'emek, Israel, in order to

25   discard red-blood cells) centrifuged and resuspended with DMEM containing 10 % FCS, Pen-Strep-Nystatin mixture (100 U/ml:100 µg/ml:1.25 un/ml) and L- Glutamin. Washed cells were then seeded in a sterile tissue culture medium flask at 3-10 X 10^7 cells/flask. At the next day cells were washed with PBS to remove residual RBC and dead cells. The cells were kept at 37 °C in a tissue culture incubator under humidified

30   condition with 5 % CO_2. The medium was changed every 3 to 4 days. At 60-80 % confluence, the cells were detached from the growth flask using 0.25 % trypsin-EDTA

and seeded into new flasks. Following 2-40 passages, when cells reached 60-80 %
confluence, cells were collected for analysis or for culturing in bioreactors.

    PluriX™ Plug Flow bioreactor - The PluriX™ Plug Flow bioreactor
(Pluristem, Haifa, Israel; as illustrated in Figure 1G, see also U.S. Pat. No. 6,911,201),

5   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 (6a in Figure 1G), and

10   peristaltic pump (9 in Figure 1G). The bioreactor contains a sampling and injection
point (4 in Figure 1G), allowing the sequential seeding of cells. Culture medium was
supplied at pH 6.7-7.4 from a reservoir (1 in Figure 1G). The reservoir was supplied by
a filtered gas mixture (2, 3 in Figure 1G), containing air/CO_2/O_2 at differing
proportions, depending on cell density in the bioreactor. The O_2 proportion was suited

15   to the level of dissolved O_2 at the bioreactor exit, determined by a monitor (6 in Figure
1G). 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 (7 in Figure 1G) which enables collection of circulating,
nonadherent cells. Circulation of the medium was obtained by a peristaltic pump (9 in

20   Figure 1G). The bioreactor was further equipped with an additional sampling point (10
in Figure 1G) and containers for continuous medium exchange.

    Production of 3D-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

25   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 (see Figure 1G).
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

30   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

5   the bioreactor and bioreactor and carriers were washed 3-5 times with PBS. 3D-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.

3D-adherent cell quality biological assays – Cryopreserved 3D-adherent cells

10 were thawed and counted. For cell viability evaluation, 2 X 10^5 cells were seeded in a 150 cm^2 tissue culture flask and their adherence capability and repopulation was evaluated within 7 days following seeding. Thereafter, the 3D-adherent cell membrane marker phenotype was analyzed using fluorescence monoclonal antibodies flow-cytometer (Beckman Coulter, Fullerton, CA).

15   *Comparison between the cell membrane marker profile of 3D and 2D cultured adherent cells using flow cytometry assays* 100,000 – 200,000 adherent cells from 2D cultures and 3D flow system cultures were suspended in 0.1 ml of culture medium in a 5 ml tube and incubated (4 °C, 30 minutes, dark conditions) with saturating concentrations of each of the following MAbs: FITC-conjugated anti-human CD90

20 (Chemicon International Inc. Temecula, CA), PE conjugated anti human CD73 (Bactlab Diagnostic, Ceasarea, Israel), PE conjugated anti human CD105 (eBioscience, San Diego, CA), FITC conjugated anti human CD29 (eBioscience, San Diego, CA), Cy7-PE conjugated anti-human CD45 (eBiosience), PE-conjugated anti-human CD19 (IQProducts, Groningen, The Netherlands), PE conjugated anti human CD14 MAb

25 (IQProducts), FITC conjugated anti human CD11b (IQProducts) and PE conjugated anti human CD34 (IQProducts) or with FITC conjugated anti human HLA-DR MAb (IQProducts). Following incubation the cells were washed twice in ice-cold PBS containing 1 % heat-inactivated FCS, resuspended in 500 μl formaldehyde 0.5 % and analyzed using the FC-500 flow-cytometer (Beckman Coulter, Fullerton, CA).

30   *Comparison between the protein profile of 3D and 2D cultured adherent cells using mass spectrometry analysis* - 2D and 3D derived culturing procedures adherent cells were produced from the placenta as described above. Briefly, the 2D cultures were

produced by culturing 0.3-0.75 X 10^6 cells in 175 cm^2 flasks for 4 days under humidified 5 % CO_2 atmosphere at 37 °C, until reaching 60 - 80 % confluence. The 3D cultures were produced by seeding 2-10 x 10^6 cells/gram in a bioreactor containing 2000 carriers, and culturing for 18 days. Following harvesting, cells were washed (X

5   3) to remove all the serum, pelleted and frozen. Proteins were isolated from pellets [using Tri Reagent kit (Sigma, Saint Louis, USA) and digested with trypsin and labeled with iTRAQ reagent (Applied Biosciences, Foster City, CA)], according to the manufacturers protocol. Briefly, iTRAQ reagents are non-polymeric, isobaric tagging reagents. Peptides within each sample are labeled with one of four isobaric, isotope-

10   coded tags via their N-terminal and/or lysine side chains. The four labeled samples are mixed and peptides are analyzed with mass spectrometry. Upon peptide fragmentation, each tag releases a distinct mass reporter ion; the ratio of the four reporters therefore gives relative abundances of the given peptide in a sample. (information at: www.docs.appliedbiosystems.com/pebiodocs/00113379.pdf).

15   Proteomics analysis of 2D culture versus 3D culture of placenta derived adherent cells was performed in the Smoler proteomic center (department of Biology, Technion, Haifa, Israel) using LC-MS/MS on QTOF-Premier (Waters, San Francisco, CA), with identification and analysis done by Pep-Miner software [Beer, I., et al., Proteomics, 4, 950-60 (2004)] against the human part of the nr database. The proteins

20   analyzed were: heterogeneous nuclear ribonucleoprotein H1 (Hnrph1 GenBank Accession No. NP_005511), H2A histone family (H2AF, GenBank Accession No. NP_034566.1), eukaryotic translation elongation factor 2 (EEEF2, GenBank Accession No. NP_031933.1), reticulocalbin 3, EF-hand calcium binding domain (RCN2, GenBank Accession No. NP_065701), CD44 antigen isoform 2 precursor (GenBank

25   Accession No. NP_001001389, calponin 1 basic smooth muscle (CNN1, GenBank Accession No. NP_001290), 3 phosphoadenosine 5 phosphosulfate synthase 2 isoform a (Papss2, GenBank Accession No. NP_004661), ribosomal protein L7a (rpL7a, GenBank Accession No. NP_000963) and Aldehyde dehydrogenase X (ALDH X, GenBank Accession No. P47738). Every experiment was done twice. Because of the

30   nature of the analysis, every protein was analyzed according to the number of peptides of which appeared in a sample (2-20 appearances of a protein in each analysis)

Comparison between secreted proteins in 3D and 2D cultured adherent cells using ELISA - 2D and 3D derived culturing procedures adherent cells produced from the placenta, were produced as described above, with 3D cultures for the duration of 24 days. Conditioned media were thereafter collected and analyzed for Flt-3 ligand, IL-6,
5   Thrombopoietin (TPO) and stem cell factor (SCF), using ELISA (R&D Systems, Minneapolis, MN), in three independent experiments. Results were normalized for 1 X 10^6 cells / ml.

Osteoblast differentiating medium- Osteogenic differentiation was assessed by culturing of cells in an osteoblast differentiating medium consisting DMEM
10   supplemented with 10 % FCS, 100 nM dexamethasone, 0.05 mM ascorbic acid 2-phosphate, 10 mM B-glycerophosphate, for a period of 3 weeks. Calcified matrix was indicated by Alizzarin Red S staining and Alkaline phosphatase was detected by Alkaline phosphatase assay kit (all reagents from Sigma- Aldrich, St. Lewis, MO).

Experimental Results

15   ***The PluriX™ Bioreactor System creates a physiological -like microenvironment.***

In order to render efficient culture conditions for adherent cells, a physiological - like environment (depicted in Figure 1A) was created artificially, using the PluriX Bioreactor (Pluristem, Haifa, Israel; carrier is illustrated in Figure 1G and shown before
20   seeding in Figure 1B). As is shown in Figures 1C-F, bone marrow produced 3D-adherent cells were cultured successfully and expanded on the 3D matrix, 20 days (Figures 1B-C, magnified X 150 and 250 respectively) and 40 days (Figures 1C-D, magnified X 350 and 500 respectively) following seeding.

Cells grown in the PluriX Bioreactor system were significantly expanded -
25   Different production lots of placenta derived 3D-adherent cells were grown in the PluriX bioreactor systems. The seeding density was 13,300 cells/carrier (to a total of 2 X 10^6 cells). Fourteen days following seeding, cell density multiplied by 15 fold, reaching approximately 200,000 cells/carrier (Figure 2), or 30 X 10^6 in a bioreactor of 150 carriers. In a different experiment, cells were seeded into the bioreactor at density
30   of 1.5 X 10^4 cells/ml and 30 days following seeding the carriers contained an over 50-fold higher cell number, i.e. approx. 0.5 X 10^6 cells/carrier, or 0.5 X 10^7 cells/ml. The cellular density on the carriers at various levels of the growth column was consistent,

indicating a homogenous transfer of oxygen and nutrients to the cells. The 3D culture system was thus proven to provide supporting conditions for the growth and prolonged maintenance of high-density mesenchymal cells cultures, which can be grown efficiently to an amount sufficient for the purpose of supporting engraftment and

5   successful transplantation.

3D-adherent cells show unique membrane marker characteristics – In order to define the difference in the secretion profile of soluble molecules and protein production, effected by the bone environment mimicking 3D culturing procedure, FACs analysis was effected. As is shown in Figure 3A, FACS analysis of cell markers depict

10   that 3D-adherent cells display a different marker expression pattern than adherent cells grown in 2D conditions. 2D cultured cells expressed significantly higher levels of positive membrane markers CD90, CD105, CD73 and CD29 membrane markers as compared to 3D cultured cells. For example, CD105 showed a 56 % expression in 3D cultured cells vs. 87 % in 2D cultured cells. Adherent cells of both 2D and 3D placenta

15   cultures, did not express any hematopoietic membrane markers (Figure 3B).

3D-adherent cells show a unique profile of soluble factors – The hematopoietic niche includes supporter cells that produce an abundance of cytokines, chemokines and growth factors. In order to further define the difference between 2D and 3D cultured adherent cells, the profile of the four main hematopoietic secreted proteins in the

20   conditioned media of 2D and 3D adherent cell cultures was effected by ELISA. Figures 4A-C show that cells grown in 3D conditions produced condition media with higher levels of Flt-3 ligand (Figure 4A), IL-60 (Figure 4B), and SCF (Figure 4C), while low levels of IL-6, and close to zero level of Flt-3 ligand and SCF, were detected in the condition media of 2D cultures. Production of Trombopoietin (TPO) was very low and

25   equal in both cultures.

3D-adherent cells show a unique protein profile in mass spectrometry analysis – In order to further define the difference between 2D and 3D cultured adherent cells, the protein profile of these cells was analyzed by mass spectrometry. Figure 4D shows that 2D and 3D cultured adherent cells show a remarkably different protein expression

30   profile. As is shown in Table 1 below, 3D cultured cells show a much higher expression level of H2AF and ALDH X (more than 9 and 12 fold higher, respectively) and a higher level of the proteins EEEF2, RCN2 and CNN1 (ca. 3, 2.5 and 2 fold, respectively). In

addition, 3D cultured cells show ca. half the expression levels of the proteins Hnrph1 and CD44 antigen isoform 2 precursor and ca. a third of the expression levels of Papss2 and rpL7a.

Table 1

protein	Protein level (relative to iTRAQ reporter group)			
	2D cultured adherent cells		3D cultured adherent cells	
	Av	*SD*	*Av*	*SD*
Hnrph1	1.434493	0.260914	0.684687	0.197928
H2AF	0.203687	0.288058	1.999877	0.965915
EEEF2	0.253409	0.130064	0.799276	0.243066
RCN2	0.54	0.25	1.34	0.26
CD44 antigen isoform 2 precursor	1.68	0.19	0.73	0.17
CNN1	0.77	0.15	1.55	0.17
Papss2	1.48352	0.314467	0.45627	0.137353
rpL7a	1.22	0.24	0.43	0.05
ALDH X	0.15847	0.22411	1.986711	0.212851

3D-adherent cells have the capacity to differentiate into osteoblasts - In order to further characterize 3D-adherent cells, cells were cultured in an osteoblast differentiating medium for a period of 3 weeks. Thereafter, calcium precipitation was effected. Differentiated cells were shown to produce calcium (depicted in red in Figures 5A-B) whereas control cells maintained a fibroblast like phenotype and demonstrated no mineralization (Figures 5C-D). These results show that placenta derived 3D-adherent cells have the capacity to differentiate *in vitro* to osteoblasts cells.

EXAMPLE 2

ASSESSMENT OF THE ABILITY OF PLACENTA DERIVED 3D-ADHERENT CELLS TO IMPROVE HSC ENGRAFTMENT

3D-adherent cell's support of HSC engraftment was evaluated by the level of human hematopoietic cells (hCD45+) detected in sub lethally irradiated or chemotherapy pretreated immune deficient NOD-SCID mice.

Materials and Experimental Procedures

Isolation of CD34+ Cells - Umbilical cord blood samples were taken under sterile conditions during delivery (Bnei Zion Medical Center, Haifa, Israel) and

mononuclear cells were fractionated using Lymphoprep (Axis-Shield PoC As, Oslo, Norway) density gradient centrifugation and were cryopreserved. Thawed mononuclear cells were washed and incubated with anti-CD34 antibodies and isolated using midi MACS (Miltenyl Biotech, Bergish Gladbach, Germany). Cells from more than one

5   sample were pooled for achieving the desired amount (50,000-100,000 cells).

Detection of transplanted cells in irradiated mice - Seven week old male and female NOD-SCID mice (NOD-CB17-Prkdcscid/J; Harlan/ Weizmann Inst., Rehovot Israel) were maintained in sterile open system cages, given sterile diets and autoclaved acidic water. The mice were sub lethally irradiated (350 cGy), and thereafter (48 hr

10   post irradiation) transplanted with 50,000-100,000 hCD34$^+$ cells, with or without additional adherent cells (0.5 x 10^6 - 1 x 10^6) derived from placenta or adipose tissue (3-7 mice in each group), by intravenous injection to a lateral tail vein. Four to six weeks following transplantation the mice were sacrificed by dislocation and BM was collected by flushing both femurs and tibias with FACS buffer (50 ml PBS, 5 ml FBS, 0.5 ml

15   sodium azid 5 %). Human cells in the mice BM were detected by flow cytometry, and the percentage of the human and murine CD45 hematopoietic cell marker expressing cells in the treated NOD-SCID mice was effected by incubating cells with anti-human CD45-FITC (IQ Products, Groningen, The Netherlands). The lowest threshold for unequivocal human engraftment was designated at 0.5 %.

20   *Detection of transplanted cells in mice treated with chemotherapy* - 6.5 week old male NOD-SCID mice (NOD.CB17/JhkiHsd-scid; Harlan, Rehovot Israel), maintained as described hereinabove for irradiated mice, were injected intraperitoneally with Busulfan (25 mg/kg- for 2 consecutive days). Two days following the second Busulfan injection, mice were injected with CD34+ cells alone, or together with 0.5 X

25   10^6 adherent cells, produced from the placenta. 3.5 weeks following transplantation, mice were sacrificed, and the presence of human hematopoietic cells was determined as described hereinabove for irradiated mice.

Experimental Results

3D-adherent cells improved engraftment of HSC in irradiated mice - Human

30   CD34+ hematopoietic cells and 3D-adherent cells derived from placenta or adipose tissues were co-transplanted in irradiated NOD-SCID mice. Engraftment efficiency was evaluated 4 weeks following co-transplantation, and compared to mice transplanted

with HSC alone. As is shown in Table 2, co-transplantation of 3D-adherent cells and UCB CD34+ cells resulted in considerably higher engraftment rates and higher levels of human cells in the BM of recipient mice compared to mice treated with UCB CD34+ cells alone.

Table 2

Transplanted cells	Average h-CD45	STDEV
CD34	3.8	7.9
CD34 + 3D-adherent cells from placenta	5.1	12.2
CD34 + 3D-adherent cells from adipose	8.7	9.6

3D-adherent cells improved engraftment of HSC in mice treated with chemotherapy - Human CD34+ hematopoietic cells were co-transplanted with 500,000-2D-adherent cells or 3D-adherent cells derived from placenta, into NOD-SCID mice pretreated with chemotherapy. Engraftment efficiency was evaluated 3.5 weeks following co-transplantation, and compared to mice transplanted with HSC alone. As is shown in Table 3 and Figure 6, co-transplantation of adherent cells and UCB CD34+ cells resulted in higher engraftment levels in the BM of the recipient mice compared to UCB CD34+ cells alone. Moreover, as is shown in Table 3, the average level of engraftment was higher in mice co-transplanted with placenta derived adherent cells grown in the PluriX bioreactor system (3D-adherent cells) than in the mice co-transplanted with cells from the same donor, grown in the conventional static 2D culture conditions (flask).

Table 3

Transplanted cells	Average h-CD45	STDEV
CD34	0.9	1.1
CD34 + conventional 2D cultures from placenta	3.5	0.2
CD34 +3D-adherent cell from placenta	6.0	7.9

FACS analysis results shown in Figures 7A-B demonstrate the advantage of co-transplanting adherent cells with hHSCs (Figure 7B), and the ability of adherent cells to improve the recovery of the hematopoietic system following HSC transplantation.

Taken together, these results show that adherent cells may serve as supportive cells to improve hematopoietic recovery following HSCs transplantation (autologous or allogenic). The ability of the 3D-adherent cells to enhance hematopoietic stem and/or progenitor cell engraftment following HSCs transplantation may result from the 3D-

5   adherent cell ability to secrete HSC supporting cytokines that may improve the homing, self-renewal and proliferation ability of the transplanted cells, or from the ability of those cells to rebuild the damaged hematopoietic microenvironment needed for the homing and proliferation of the transplantable HSCs

10   *EXAMPLE 3*

THE SUPPRESSION OF LYMPHOCYTE RESPONSE BY 2D AND 3D CULTURED ADHERENT CELLS

Adherent cells, and particularly 3D-adherent cells, were found to suppress the immune reaction of human cord blood mononuclear cells in an MLR assay

15   *Materials and Experimental Procedures*

Mixed lymphocyte reaction (MLR) assay – The immunosuppressive and immunoprivileged properties of 2D and 3D derived culturing procedures adherent cells produced from the placenta, were effected by the MLR assay, which measures histocompatibility at the HLA locus, as effected by the proliferation rate of

20   incompatible lymphocytes in mixed culturing of responsive (proliferating) and stimulating (unproliferative) cells. Human cord blood (CB) mononuclear cells (2×10^5) were used as responsive cells and were stimulated by being co-cultured with equal amounts (10^5) of irradiated (3000 Rad) human peripheral blood derived Monocytes (PBMC), or with 2D or 3D cultured adherent cells, produced from the placenta, or a

25   combination of adherent cells and PBMCs. Each assay was replicated three times. Cells were co-cultured for 4 days in RPMI 1640 medium (containing 20 % FBS under humidified 5 % CO_2 atmosphere at 37 °C), in a 96-well plate. Plates were pulsed with 1 μC ^{3}H-thymidine during the last 18 hours of culturing. Cells were then harvested over fiberglass filter and thymidine uptake was quantified with a scintillation counter.

30   *Experimental Results*

Figure 8A shows the immune response of CB cells as represented by the elevated proliferation of these cells when stimulated with PBMCs, which, without being bound

by theory, is probably associated with T cell proliferation in response to HLA
incompatibility. However, a considerably lower level of immune response was
exhibited by these cells when incubated with the adherent cells of the invention.
Moreover, the CB immune response to PBMCs was substantially reduced when co-
incubated with these adherent cells. Thus, in a similar manner to MSCs, adherent cells
were found to have the potential ability to reduce T cell proliferation of donor cells,
typical of GvHD. Although both cultures, 2D and 3D, reduced the immune response of
the lymphocytes, and in line with the other advantages of 3D-adherent cells described
hereinabove, the 3D adherent cells were more immunosuppressive.

EXAMPLE 4

3D ADHERENT CELLS MANUFACTURED BY PLURIX COMPARED TO 3D ADHERENT CELLS MANUFACTURED BY CELLIGEN

In order to provide large scale 3D adherent cells, a new manufacturing system
was utilized referred to herein as Celligen.

Materials and Experimental Methods

PluriX™ Plug Flow bioreactor – As described in Example 1, hereinabove.

Production of 3D-adherent cells by Plurix (PLX cells) – As described in
Example 1, hereinabove.

Celligen™ Plug Flow bioreactor – The production of adherent cells by
Celligen™ (PLX-C cells) is composed of several major steps as illustrated in Figure 8B.
The process starts by collection of a placenta from a planned cesarean delivery 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 1-3 weeks 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 (IPC1).

Immediately after taking the placenta from the donor (during the caesarean procedure), it was placed in a sterile plastic bag and then in a Styrofoam box with ice packs. The placenta was delivered and immediately placed in a quarantine area until released to use by Quality Control (QC) and Quality Assurance (QA). All the following production steps were performed in a quarantine, clean room facility until QC approval of mycoplasma test results arrived and the cells were release for 2D cell growth.

Recovery and Processing of adherent cells

To initiate the process, the whole placenta was cut into pieces under aseptic conditions under laminar flow hood, washed with Hank's buffer solution and incubated for 3 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 gentamycine 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 pipeting, suspended cells were then washed with 2D-Medium supplemented with antibiotics, seeded in 80 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, cells were transferred to the 2D production clean room (2DP). Once in Room 2DP, culture was continued for another 3-5 passages. IPC-3 sample was taken for immune phenotype after passage 4. Throughout the process, cultures were grown in 2D-Medium without antibiotics in a tissue culture incubator under humidified conditions with 5 % CO2 at 37 °C. After a total of 6-8 passages (9-16 cell doublings), cells were collected and cryopreserved as the 2D-Cell Stock (2DCS).

The first passage was usually carried out after 10-15 days. Beginning at passage 2 and continuing until passage 6-8, cells were passaged when the culture reached 70-80 % confluence, usually after 3-5 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 $3 \pm 0.2 \times 10^3$ cells/cm^2. The size of the tissue culture flasks raised as the passages proceed. The culturing process started in 80 cm^2 tissue culture flask, continued in 175 cm^2, then in 500 cm^2 (Triple flask) and finally the cells were seeded

5   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

10   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

15   $1.5 - 2.5 \times 10^9$ cells were manufactured from one placenta. 4 ml of the cells were stored at a final concentration of 10×10^6/ ml in 5ml 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 located in the Cold Storage Room. This material

20   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 ($150 \pm 30 \times 10^6$) 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

25   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

30   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 3D-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 8C. 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, $150 \pm 30 \times 10^6$ 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 $\pm$ 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 $\pm$ 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-X Cells from the Bioreactor

The cell harvest process started at the end of the growth phase (4-10 days). Two
5   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, and the other one was 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 located in the Cold Storage Room, in case a repeat
10   Mycoplasma testing was needed. These medium samples were 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:
15   The bioreactor vessel was emptied using gravitation via tubing to a waste container. The vessel was opened, by removing the head plate, and the carriers were aseptically transferred, using sterile forceps, from the basket to the upper basket net (see Figure 8C). The bioreactor vessel was then closed and refilled with 1.5 L pre-warmed PBS (37 °C). The agitation speed was increased to 150 RPM for 2 minutes. The PBS
20   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 5 minutes in 150 RPM, 37 °C. Cell suspension was collected to a 5 L
25   sterile container containing 250 ml FBS. Cell suspension was divided to 4 500 ml sterile centrifuge tubes and a Mycoplasma test sample was withdrawn. Closed centrifuge tubes were transferred through the 3DP active pass-through into the class 10,000 filling room (FR1) in which the cells were aseptically filled and cryopreserved as PLX-C.

Cell Cycle analysis - PLX-C cells obtained by Celligen and PLX cells obtained
30   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
5   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
10   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
15   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-
20   conjugated anti-human CD86 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
25   (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
30   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

5   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 %

10   CO_2 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

15   several major differences (summarized in Table 4, below).

Table 4: Comparison between Plurix system and Celligen system

Parameter	Cell growth according to Example 1	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
Bed configuration	Conic, 50 ml column	Cylinder Packed bed	Present teachings - Better flow of medium and nutrients. Teachings of Example 1 – Inefficient flow due to narrow outlet form of the

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Parameter	Cell growth according to Example 1	Teachings of the present invention	Improvement
			conic structure Better homogeneity of medium flow. Channeling in the teachings of Example 1.
Cell concentration at seeding (cell / gr carrier)	3×10^6 cell / gr carrier	5×10^6 cell / gr carrier	Better cell to cell interaction in the present teachings
Cell concentration at seeding (cell / ml)	0.015×10^6 cell / ml	0.1×10^6 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	Teachings of Example 1 - 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	4-10 days	Better product quality. Efficient harvest process. Better yield. Lower cost process in the present teachings
Mode of	Repeated	Perfusion	Present

Parameter	Cell growth according to Example 1	Teachings of the present invention	Improvement
operation	batch – medium change twice a week	mode – rate was adjusted according to the glucose concentration (the medium was changed at glucose concentration of 550 ± 50 mg/L)	teachings - Moderate 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

Parameter	Cell growth according to Example 1	Teachings of the present invention	Improvement
			Improves other control loops (temp., DO, pH)
Temperature control	The production was carried out inside an incubator. Indirect temperature control (of the incubator chamber). Heat transfer via air interface	On-line direct control. Heat transfer via water jacket.	Present teachings - More accurate measurement of the culture temperature. Quick response. Short time to 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	On-line Control and monitoring	Present teachings - Better control of pH level.

Parameter	Cell growth according to Example 1	Teachings of the present invention	Improvement
	medium)		Better maintenance of a specified working conditions
Aeration	Sparge only	Overlay (sparge as an option)	Teachings of Example 1 - Aeration by sparge creates foam that might damage the cells.

The changes in the manufacturing process resulted in changes in characteristics of the obtained 3D 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 9A-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 9A). 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 5, below).

Table 5: Gene expression in Celligen compared to Plurix cells

Gene	Celligen vs. Plurix (fold change)	p-value(treat)
interferon-induced protein with tetratricopeptide repeats	17.52	0.0401812
aldchyde 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, homolog (S. cere	5.05	0.00276524
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 repeats	2.24	0.0177928
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.	2.23	0.00294705

cerev

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	**-3.13**	5.59E-05

gelatinase,		
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, Drosophila)	-3.62	0.00821813
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 and HLA-DR (data not shown). 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 10A-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 10C).

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 11A-C). Moreover, PLX-C expressed low levels HLA class I as detected by staining for HLA A/B/C (Figure 11D). The expression of stimulatory and co-stimulatory molecules was similar to bone marrow (BM) derived MSCs (as shown in Figures 11A-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 12A-B, PLX-C cells both escape allorecognition, and reduce T cell response, as measured by Thymidine incorporation. Furthermore, the reduction in
5   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 12B) and Phytohemagglutinin (PHA), and non-specific stimulation by anti-CD3, anti-CD28 (data not shown).

10   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
15   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
20   mechanism of action of PLX-C. As depicted in Figures 13A-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
25   secretion level of TNFα decreased, in a dose dependent manner (Figure 13C).

EXAMPLE 5

BIODISTRIBUTION OF PLX-C

Materials and Experimental Methods

30   *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 14).

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 CO_2 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 % CO_2 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, CXL cells continued to divide following infection, and expression levels of luciferase in the growing cells remained strong and stable (Figure 15).

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 16A-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. CXL 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.

EXAMPLE 6

ADHERENT CELLS ARE CAPABLE OF TREATING LIMB ISCHEMIA IN VIVO

To determine whether implantation of placental derived adherent cells can reduce ischemic damage and improve clinical and motor functions, the hind limb ischemia model was used, as follows.

Materials and Experimental Methods

Hind limb ischemia model - Hind limb ischemia was induced in 20 Male Balb/c mice, which are not immuno-deficient, at the age of 8-10 weeks, body weight approximately 25 g ± 20 %. Animals handling was according to the National Institute of Health (NIH) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Animals were housed under standard laboratory conditions. Animals were kept in a climate controlled environment. Temperatures range was between 20 – 24 °C and relative humidity (RH) was between 30 – 70 % with 12 hours light and 12 hours dark cycle.

Animals were randomized using a computer generated randomization program "Research Randomizer" and divided into 2 groups of 10 animals. One group received intramuscular (IM) injection of 1 x 10^6 placental derived adherent cells (PLX-C) cells and the other group served as control and was injected with PBS.

Surgical Procedures - 1-1.5 cm incision was made in the skin in the inguinal area. The femoral artery was ligated twice with 6-0 silk and transected distal to the ligature. The wound was closed with 3-0 silk and the mice were allowed recovering. Five hours post-operative excision of one femoral artery, mice received an IM injection of 1 x 10^6 placental derived adherent cells (PLX-C) in a total volume of 50 µl at 2 administration Sites. Control group animals were identically injected with PBS (Gibco), see Table 6 below.

Table 6: Pilot study of PLX-C in Mouse Hind limb Ischemia Model

Test Group	Treatment	No. of Mice per group	Cell Dose	Lot	Time of Sacrifice Post-dosing 21 days
1	PLX-C i.m	n=10 ♂	1x10^6	C.G.13.0	n=10 ♂
2	PBS i.m	n=10 ♂	0	N/A	n=10 ♂

Follow up - Blood flow on legs from both sides was measured 3 consecutive times with a non contact laser Doppler just after the operation, and on days 6, 9, 14 and 21 post operation, and is expressed as the ratio of the flow in the ischemic limb to that in the normal limb [Tokai. J. et al].

Macroscopic evaluation of ischemic severity - The ischemic limb was macroscopically evaluated at days 1, 6, 9, 14, 21, till study termination by using graded morphological scales for necrotic area; grade 0: absence of necrosis, grade I: necrosis limiting to toes (toes loss), grade II: necrosis extending to a dorsum pedis (foot loss), grade III: necrosis extending to a crus (knee loss), grade IV: necrosis extending to a thigh (total hind-limb loss) [Tokai. J. et al].

In vivo assessment of limb function and Ischemic damage - Semiquantitative assessment of impaired use of the ischemic limb was be performed serially as follows: 3 = dragging of foot, 2 = no dragging but no plantar flexion, 1 = plantar flexion, and 0 = flexing the toes to resist gentle traction of the tail (Rutherford et al., 1997).

Molecular and biochemical analysis - In addition to clinical evaluation, molecular and biochemical samples were obtained at day 21 and are currently being

analyzed in order to better understand the molecular mechanisms underlying improved healing in the placental derived adherent cells (PLX-C) injected group.

Experimental Results

Implantation of placental derived adherent cells significantly induces blood
5 ***flow in the hip and foot of the ischemic hind limb model*** – To test the efficacy of the adherent cells *in vivo*, mice were subjected artery ligation followed by intramuscular injections of placental derived adherent cells and the blood flow was measured in the hips and foot (both body sides) using a non contact laser Doppler at pre-determined time period following treatment. As is shown in Figure 17, injection of PLX-C
10 markedly improved blood flow (BF) to the damaged limb, as determined by blood flow assessments, increase in limb function, increase in capillary density, decrease in oxidative stress and endothelial damage. In terms of blood flow, the effect was demonstrated 9 days following injection and was observed throughout the entire study. In the PLX-C treated group, BF increased from 24 ± 2.3 to 80 ± 4.7 %, while in the
15 control, vehicle-treated group BF was in the range of 35 ± 2 to 54 ± 4.5 % - in the hip/implantation area (day 0 vs. day 21, respectively). Similarly to the hip area, but to a lesser extent, an increase in BF was also demonstrated in the paw area of PLX-C treated mice. Thus, in the vehicle treated group BF increased from 12 ± 0.6 to 46 ± 4.9 %, while in the PLX-C group BF increased from 10 ± 0.7 to 52 ± 5.5 % (day 0 vs. day 21
20 respectively), as shown in Figure 17.

The adherent cells are capable of improving limb function in vivo – To further evaluate the *in vivo* effects of the placental derived adherent cells, the limb functions in the treated mice were assessed using the scoring system described under Materials and Experimental Methods, hereinabove. As is shown in Figure 18, mice treated with the
25 adherent cells exhibited a significant improvement in the limb function (2.5 ± 0.2 vs. 2.1 ± 0.2 control vs. PLX-C group, respectively, note the significant effect at day 21 post treatment). However, the degree of improvement in limb function during the 21 days observation was comparable, suggesting that PLX-C, under the conditions of the present study did not exhibit a major change of function recovery.

30 Macroscopic assessment of ischemic severity revealed that in the control, vehicle treated group, necrosis limited to the toes was observed in two animals on day 6. In the PLX-C treated group, necrosis limited to the toes, was demonstrated only in one

animal and only after 14 days. Post mortem immunohistochemical analyses of the limbs treated with PLX-C indicated a significant increase in the number of new capillaries (vessels) supplying the limb and suggesting PLX-C possess the ability to promote angiogenesis (Figure 19).

5   Finally, a decreased oxidative stress and a reduction in endothelial inflammation (which was a surrogate parameter for improved endothelial function) in the treated animals were observed in the PLX-C treated mice (Figures 20A-B). This was likely due to increased oxygen supply in mice treated with PLX-C cells, but not in control mice treated with PBS.

10   In conclusion, when compared to control, PBS-injected mice, none of the PLX-C injected mice exhibited any adverse clinical signs or symptoms in response to intra mascular (i.m.) cell administration. Thus, PLX-C induces an increase in blood flow, likely resulting from angiogenesis as supported by histological evaluation of the damaged limb. In addition, the delay in development of necrosis and the difference in

15   number of affected animals are suggestive of a clinical response.

Implantation of placental derived adherent cells

Another efficacy study was carried out in Balb/C mice comprising safety endpoints (i.e., gross necropsis and histopathological analysis of selected organs) as was described in the materials and methods section above.

20   In this study, seven groups of mice, each consisting of 10 male Balb/c mice (Ischemic hind limb) were used as detailed in Table 7, hereinbelow. A single group of 10 mice did not have ischemia induced (in order to test the overall safety and tolerability of PLX-C cells in normal, healthy animals). Following induction of ischemia, control buffer or PLX-C cells were administered i.m. to the affected limb, and

25   mice were observed for up to 1 month post dosing. A single group of mice received two separate injections in the affected limb, separated by 1 week (Days 1 and 8). Blood flow was monitored by Laser Doppler analysis, and ischemic severity was assessed macroscopically and behaviorally out to 30 days post-dose, at which time, mice were sacrificed, and tissues retained for histological analysis.

30

Table 7: Efficacy study of PLX-C in Mouse Hind limb Ischemia Model

Test Group no.	Treatment	Cell Amount (Dose)	Number of Treatments	Lot	Time of Sacrifice Post-dosing 30 days
1	PLX-C	1×10^6	1	C.G.13.0	10 ♂
2	PLX-C	1×10^6	1	C.G.25.0	10 ♂
3	PLX-C	1×10^6	2	C.G.25.0	10 ♂
4	PLX-C	0.5×10^6	1	C.G.13.0	10 ♂
5	PLX-C	0.1×10^6	1	C.G.13.0	10 ♂
6	Control Freezing Medium	N/A	1	N/A	10 ♂
7*	PLX-C	1×10^6	1	C.G.13.0 C.G.25.0	10 ♂

In this study, different PLX-C batches at three concentrations were administered. The results showed that 0.1×10^6 and 0.5×10^6 PLX-C had a minor therapeutic benefit. A noticeable improvement in blood flow was observed by day 29 (end of experiment) in animals treated with 1×10^6. This improvement in blood flow was significant ($p < 0.05$) in group 2M (batch G.C25) in comparison to control, vehicle injected mice. Additionally, a second injection of the same batch of cells significantly improved BF on day 15 compared to the single injection (55 ± 24 compared to 31 ± 12.9 and $27 \pm 12.5\%$, respectively). Macroscopic assessment of ischemic severity revealed that there was a trend for improvement in the groups receiving 1×10^6 (1M & 2M) in comparison to the control vehicle treated group (6M).

Altogether, these results demonstrate the efficacy of the adherent cells in inducing vascularization (e.g., blood flow) and improving limb function in hind limb ischemic mice model and suggest the use of these cells (e.g., placental derived adherent cells) for treating ischemic limb diseases.

EXAMPLE 7

PLX-C FOR THE TREATMENT OF STROKE

The aim of this study was to evaluate the therapeutic efficacy of systemic (intravenous) human PLX-C - placenta derived adherent cells transplantation in the treatment of stroke.

Materials and Experimental Methods

Subjects, surgery and transplantation

Male spontaneously hypertensive rats, suffering from hypertension, hypercholesterolemia, diabetes and microangiopathies were used. The animals were kept under constant conditions concerning temperature, air humidity and light/dark cycle. Subjects were assigned experimental groups randomly (see Table 8, below).

Table 8: Rat treatment groups in the treatment of stroke

Group no.	Treatment	No. of animals
1	PLX-C, batch 1 single administration	N=8
2	PLX-C, batch 1 double administration	N=7
3	PLX-C, batch 2 single administration	N=8
4	PLX-C, batch 2 double administration	N=7
5	Control- Vehicle solution	N=12

Animals received a single or double dose of 1×10^6 PLX-C of different batches. All transplantation procedures were conducted intravenously. The double injected group was transplanted 10 and 24 hours following brain ischemia, while single transplantations were performed 24 hours upon stroke. All transplanted cells were pre-labeled with the fluorescence dye PKH26.

Experimental brain ischemia was conducted via permanent occlusion of the right cerebral artery. Of note, one animal died following anesthesia.

Magnetic resonance investigation (MRI)

MRI of lesion development was carried out on days 1, 8, 29 and 60 using a 1.5T scanner (Philips). Infarct volumetry and brain atrophy was measured and calculated as means of values obtained by three blinded investigators using coronal T2- sequences.

Behavioral tests

Functional changes were measured by using two dependent behavioral test arrays. The Beam Walk test is a common test used to quantify sensory-motor deficits. The rats were conditioned to run across a horizontal mounted bar with the rat's home cage at the end. The time of transit was measured for five times and documented as diurnal mean value. Hanging at the beam was assessed with 20 seconds and falling down with 30 seconds. Measurements took place daily within the first week and every seventh day until the end of the observation period.

The second test, the modified neurological severity score (mNSS) contained additional sensory, motor and reflex items. Outcome of the mNSS was expressed as a score between 1 and 18, whereas points between 1 and 6 implied a mild, 7 to 12 a moderate and 13 to 18 a sever injury. Evaluation of mNSS score was performed on days 1, 4, 7, 14, 21, 28, 35, 42, 49 and 56 following brain ischemia.

Histology

Subsequent to the end of the experimental period, all rats were sacrificed and transcardially perfused with 4 % formalin solution. Extracted brains were cryopreserved and cut into 30 µm thick sections. For the appraisal of glia reaction, an immunohistochemical investigation was performed with primary antibody against GFAP. A 750 µm broad area was examined (semi-quantitatively) close to the infarct border for density of GFAP+ cells. For inspection of astroglial reactivity, 15 regions where included with average interspaces of 0.6 mm.

Statistics

All data concerning weight, MRI analysis and histological examinations were investigated for Gaussian distribution and analyzed for statistically significant differences using the ANOVA and accordingly the ANOVA on ranks.

Data collected in the Beam Walk and mNSS test were subjected to detailed statistical analysis making allowance for repeated measurements of subjects as well as for temporal development of subjects individually (stratified analysis). For balancing of inter- individual differences concerning the degree of brain damage a random intercept model was used. The data collected within the Beam Walk test therefore had to be transformed to a categorical system. Here, time values of less than 5 seconds were considered as category (0), 5 to 10 seconds as category (1), 10 to 15 seconds as category (2), 15 to 20 seconds as category (3), hanging as category (4) and falling as category (5).

Experimental Results

Weight

Periodic weighing allowed a good estimation of the subject's general state of health. An initial reduction of weight was observed in all groups due to anesthesia and the surgical intervention (data not shown). Subsequently, a quick normalization of weight and a stable course until the end of the experiment at day 60 was observed (data

not shown). The experimental groups showed a homologous progression of body weight.

Beam Walk test

All experimental groups showed a significant reduction of Beam Walk
5   categories during the course of the experiment (data not shown). A significant lower decrease of Beam Walk categories were observed in the experimental group 1 (PLX-C batch 1 single administration) compared to the control group (-0.01247 vs. -0.02931, respectively). There was no evidence for statistically significant differences between the experimental group 3 (PLX-C batch 2 single administration) and the control group
10   (data not shown).

Modified Neurological Severity Score (mNss)

All experimental groups displayed a significant reduction of neurological score points (data not shown). Comparing the mNSS results of subjects treated with PLX-2 (PLX-C double administration) revealed a statistically significant superiority compared
15   to the control group. The duplicate transplantation of batch2 (group 3) showed a significant improvement in the mNSS test compared to the simple injection of the same batch (data not shown).

Infarct volumetry

Magnetic resonance imaging is a highly sophisticated method to estimate the
20   degree of brain damage and tissue lost in vivo. Taking inter-individual fluctuations into consideration, the development of infarct volume was denoted as percentage of the infarct volume at day 1, individually. The infarct volume on day 1 did not differ significantly between the experimental groups. The general development of infarct volume displayed an approximate decrease of 50 % between day 1 and day 8. This was
25   mainly due to a retrogression of the initial brain edema. Examination of lesion development in vivo using MRI revealed that group 4 (PLX-C batch 2 double administration) subjects showed a significant reduced infarct quotient at Day 60 (0.48 ± 0.02 vs. 0.60 ± 0.03, respectively, results not shown).

Taken together, these results indicate that intravascular administration of PLX-C
30   resulted in a significant improvement of functional recovery in both behavioral tests in the treatment of stroke. Moreover, a considerable and as well statistically significant

superiority of PLX-C double transplantations was observed compared to the analogous single injection.

A corroboration of measured behavioral improvements by MRI was evident in subjects treated twice with PLX-C. In addition, a significant reduction of the infarct volume and the brain atrophy was observed at the end of the experiment. Moreover, in both functional tests a stable improvement of functional recovery following double-dosed transplantation of PLX-C was observed compared to controls and unverifiable effects upon single injections.

EXAMPLE 8

TREATMENT OF PATHOLOGIES REQUIRING CONNECTIVE TISSUE REGENERATION AND/OR REPAIR

Treating pathologies requiring bone regeneration and/or repair using the adherent cells of the invention

Animal models (e.g., Mature New Zealand white rabbits) are used to examine the effect of the adherent cells of the invention (which are derived from placenta or adipose tissue and are obtained from a 3D culture, e.g., PLX-C cells) on the healing of critical-sized segmental defects in the femora. The animals are randomly assigned to one of three groups. Animals of group A, are injected with 1-10 x 10^6 of the adherent cells (PLX-C cells) into the defect site. Animals of group B are injected with PBS. In animals of group C, the defect was left untreated. Radiographs are made immediately after the operation and at one-week intervals. At 12 weeks, the animals are sacrificed, the involved femora are removed, and undecalcified histological sections from the defects and adjacent bone are prepared. Mechanical, histological and histomorphometric studies are carried out to examine the healing of the defects and the formation of bone in and around the defected site. In addition a reverse transcription-polymerase chain reaction (RT-PCR) is performed to detect mRNA of type-I and type-II collagen.

Treating pathologies requiring tendon regeneration and/or repair using the adherent cells of the invention

Animal models (e.g., skeletally mature New Zealand white rabbits) are used to examine the effect of the adherent cells of the invention (e.g., PLX-C cells) on the

healing of tendons. The hallucis longus tendons are translated into 2.5-mm diameter calcaneal bone tunnels. The bone tunnels are treated with or without PLX-C. The animals are randomly assigned to one of three groups. Animals of group A, are injected with $1-10 \times 10^6$ PLX-C cells into the defect site or IV. Animals of group B are injected

5   with PBS. In, animals of group C the defect is left untreated. Three specimens from each group are harvested at 2, 4, and 6 weeks postoperatively and evaluation for morphologic characteristics of the healing tendon to bone interface is performed by the use of conventional histology and immunohistochemical localization of collagen Types I, II, and III.

10   *Treating pathologies requiring cartilage regeneration and/or repair using the adherent cells of the invention*

Animal models (e.g., skeletally mature New Zealand white rabbits) are used to examine the effect of the adherent cells of the invention (e.g., PLX-C cells) on the healing of cartilage. A full-thickness defect of the articular cartilage of the patellar

15   groove of the left distal femur is performed. A flap of about 6 mm is removed from the fascia overlying the quadriceps muscle and sutured to the peripheral rim of the artificial defect with 6-0 catgut. The animals are randomly assigned to one of three groups. Animals of group A are injected with $1-10 \times 10^6$ PLX-C cells into the defect site or IV. Animals of group B are injected with PBS. In animals of group C, the defect is left

20   untreated. The animals are sacrificed. Fourteen weeks after the implantation of the PLX-C cells onto the osteochondral defect, the distal femora are resected and histological evaluation are performed and the specimens are graded semiquantitatively based on the predominant nature of the repair tissue, matrix staining, regularity of the surface, structural integrity, thickness of the repair, apposition between the repaired

25   cartilage and surrounding normal cartilage, freedom from degenerative signs in repair tissue, and freedom from degenerative changes of the surrounding normal cartilage.

Treating pathologies requiring ligament regeneration and/or repair using the adherent cells of the invention

Animal models (e.g., skeletally mature New Zealand white rabbits) are used to

30   examine the effect of the adherent cells of the invention (e.g., PLX-C cells) on the healing of ligament. Unicortical circular defects of 8 mm in diameter will be performed. The animals are randomly assigned to one of three groups. Animals of group A are

injected with 1-10 x 10^6 PLX-C cells into the defect site or IV. Animals of group B are injected with PBS. In animals of group C, the defect is left untreated. The animals are sacrificed fourteen weeks after the implantation of the PLX-C cells onto the oligamental defect. Histological evaluation are performed and the specimens are graded semiquantitatively based on the predominant nature of the repaired tissue.

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.

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 and GenBank Accession numbers 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 or GenBank Accession number 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 invention.

The term "comprise" and variants of the term such as "comprises" or "comprising" are used herein to denote the inclusion of a stated integer or stated integers but not to exclude any other integer or any other integers, unless in the context or usage an exclusive interpretation of the term is required.

Any reference to publications cited in this specification is not an admission that the disclosures constitute common general knowledge in Australia.

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CLAIMS

1.   A method of treating a condition in a subject in need thereof, said method comprising the step of administering to the subject a therapeutically effective amount of adherent placental cells, wherein said condition is selected from the group consisting of ischemia, a condition requiring connective tissue regeneration and a condition requiring connective tissue repair.

2.   The method of claim 1, wherein said cells are capable of suppressing an immune reaction in the subject.

3.   The method of claim 1 or claim 2, wherein at least 10 % of said cells are at a proliferative phase.

4.   The method of any one of claims 1 to 3, wherein said cells are propagated using a two-dimensional (2D) culture.

5.   The method of any one of claims 1 to 3, wherein said cells are propagated using a three-dimensional (3D) culture.

6.   The method of claim 5, wherein said three-dimensional (3D) culture comprises a 3D bioreactor.

7.   The method of claim 5 or claim 6, wherein culturing of said cells in said three-dimensional (3D) culture is effected under perfusion.

8.   The method of claim 7, wherein said perfusion rate is adjusted in order to maintain a constant glucose concentration in the culture medium.

9.   The method of claim 8, wherein said constant glucose concentration is about 550 mg/L.

10.   The method of any one of claims 5 to 9, wherein culturing conditions of said three-dimensional (3D) culture comprise an adherent material selected from the group consisting of a polyester and a polypropylene.

11.   The method of any one of claims 1 to 10, wherein said cells comprise a positive expression marker selected from the group consisting of CD73, CD90, CD29 and CD105.

12.   The method of any one of claims 1 to 11, wherein said cells comprise a negative expression marker selected from the group consisting of CD3, CD4, CD45, CD80, HLA-DR, CD11b, CD14, CD19, CD34 and CD79.

13.   The method of any one of claims 1 to 12, wherein said cells comprise a stromal stem cell phenotype.

14.   The method of any one of claims 1 to 13, wherein said ischemia is selected from the group consisting of peripheral arterial disease (PAD) and ischemia of the central nervous system (CNS).

15.   Use of an article of manufacture comprising a packaging material, which comprises a label, for treating a condition in a subject in need thereof, wherein said condition is selected from the group consisting of ischemia, a condition requiring connective tissue regeneration and a condition requiring connective tissue repair, said packaging material packaging a pharmaceutically effective amount of adherent placental cells.

16.   The use of claim 15, wherein said cells are capable of suppressing an immune reaction in the subject.

17.   The use of claim 15 or claim 16, wherein at least 10 % of said cells are at a proliferative phase.

18.   The use of any one of claims 15 to 17, wherein said cells are propagated using a three-dimensional (3D) culture.

19.   Adherent placental cells when used for treating a condition in a subject, wherein said condition is selected from the group consisting of ischemia, a condition requiring connective tissue regeneration and a condition requiring connective tissue repair.

20.   An article of manufacture comprising a packaging material, which comprises a label, when used for treating a condition in a subject, wherein said condition is selected from the group consisting of ischemia, a condition requiring connective tissue regeneration and a condition requiring connective tissue repair, said packaging material packaging a pharmaceutically effective amount of adherent placental cells.

Date: 15 July 2013

FIG. 1A FIG. 1B

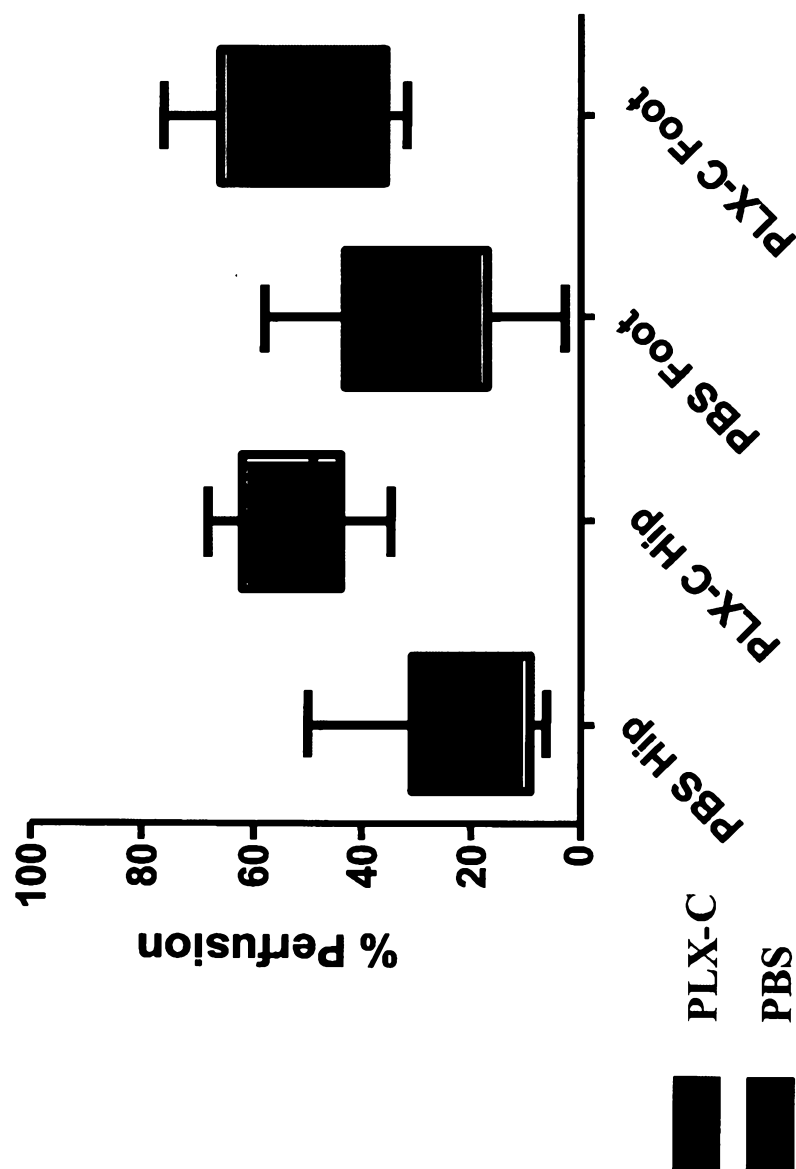FIG. 1C FIG. 1D

FIG. 1E FIG. 1F

FIG. 1G

FIG. 2

FIG. 3A

FIG. 3B

FIG. 4A

FIG. 4B

FIG. 4C

FIG. 4D

FIG. 5C

FIG. 5D

FIG. 5A

FIG. 5B

FIG. 6
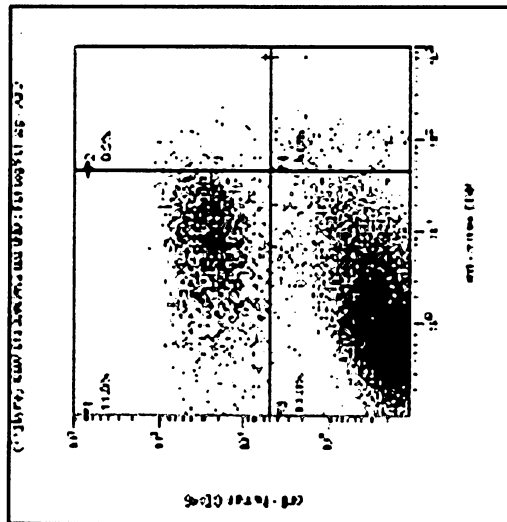
FIG. 7B

Human CD45+ (%)
Mouse CD45+ (%)

FIG. 7A

FIG. 8A

Receipt, recovery and processing of adherent cells from Human Placenta

⇨

Two Dimensional (2D) Cell Growth

⇨

Cryopreservation Procedure for 2D-Cell-Stock Product

⇨

Seeding approximately 5 x 10⁶ adherent cells in three dimensional (3D) cultures, in a Celligen bioreactor, in constant perfusion and with constant monitoring of pH, temp., glucose levels, etc.

⇨

Cell growth in bioreactor

⇨

Harvest of the 3D adherent cells in the bioreactor

⇨

Aseptic filling & cryopreservation

⇨

PLX-C Cells Storage in Liquid Nitrogen

FIG. 8B

FIG. 8C

FIG. 9A

FIG. 9B

FIG. 10C

⊟LM1: 99.54% ⊞ LM1: 0.25%

D7-Fib(FITC) IgG1(FITC)

FIG. 10B

⊟LM1: 1.24% ⊞LM1: 1.06%

KDR(FITC) IgG1(FITC)

FIG. 10A

⊟LM1: 1.52% ⊞LM1: 1.63%

CD31(FITC) IgG1(FITC)

FIG. 11A

FIG. 11B

FIG. 11C

FIG. 11D

FIG. 12A

FIG. 12B

FIG. 13A

FIG. 13B

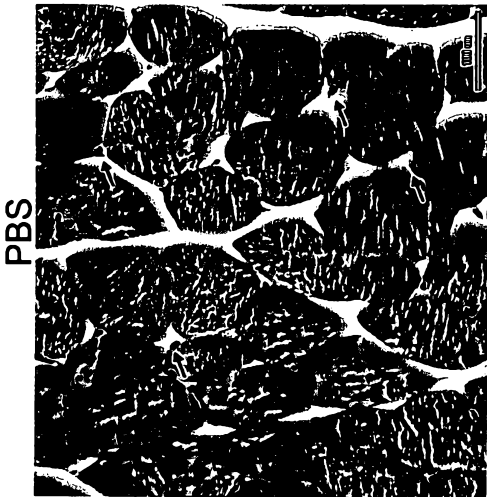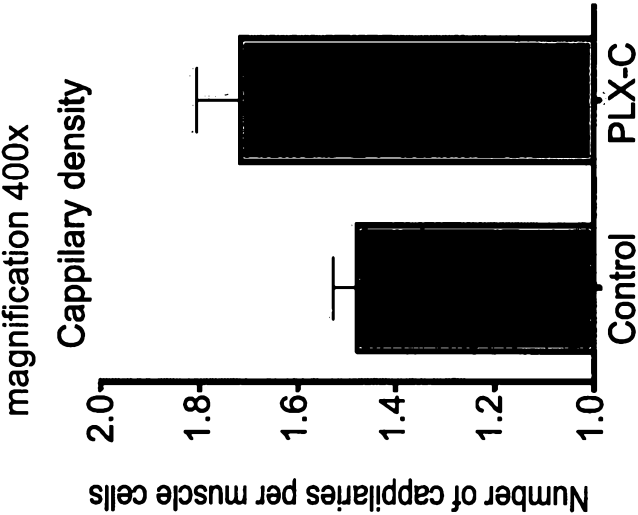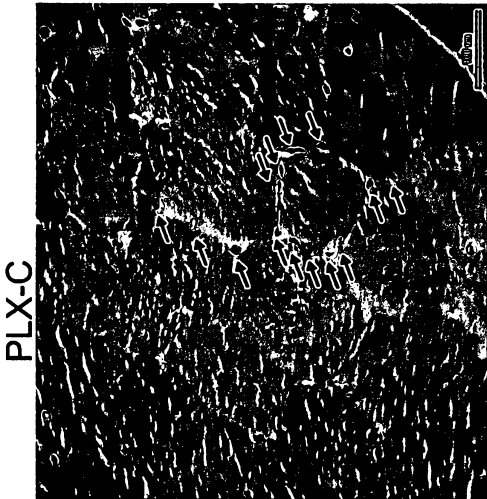FIG. 13C

FIG. 14

FIG. 15

FIG. 16B

FIG. 16A

FIG. 16D

FIG. 16C

FIG. 17

FIG. 18

FIG. 19B

FIG. 19A

FIG. 19C

FIG. 20B

FIG. 20A