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(71) Applicant *(for all designated States except US)*: **PLURISTEM LIFE SYSTEMS INC.** [IL/IL]; Matam Building # 20, 31905 Haifa (IL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **MERETZKI, Shai** [IL/IL]; 38 Raoul Wallenberg Street, 34990 Haifa (IL). **ABERMAN, Zami** [IL/IL]; 63 Ravutski Street, 43220 Raanana (IL). **BURGER, Ora** [IL/IL]; 5 Dulchin Street, 32882 Haifa (IL).

(74) Agents: **G.E. EHRLICH (1995) LTD.** et al.; 11 Menachem Begin Street, 52521 Ramat Gan (IL).

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(54) Title: METHODS FOR CELL EXPANSION AND USES OF CELLS AND CONDITIONED MEDIA PRODUCED THEREBY FOR THERAPY

(57) Abstract: A method of cell expansion is provided. The method comprising culturing adherent cells from placenta or adipose tissue under three-dimensional culturing conditions, which support cell expansion.

METHODS FOR CELL EXPANSION AND USES OF CELLS AND CONDITIONED MEDIA PRODUCED THEREBY FOR THERAPY

FIELD AND BACKGROUND OF THE INVENTION

The present invention relates to methods of cell expansion, populations of cells produced thereby and uses of same. Specifically the present invention relates to methods of expanding adherent cells from placenta or adipose tissues (along all the PCT) and therapeutic uses of same, such as for hematopoietic stem cell transplantation.

In the developing medical world a growing need exists for adult stem cells in large amounts 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.

Hematopoietic stem cells (HSCs) are precursor cells, which give rise to all the blood cell types of both the myeloid and lymphoid lineages. Engraftment and initiation of hematopoiesis by transplanted HSCs depend on those cells ability to home and proliferate within the recipient BM.

It is widely accepted that stem cells are intimately associated in vivo with discrete niches in the marrow, which provide molecular signals that collectively mediate their differentiation and self-renewal, via cell-cell contacts or short-range interactions. These niches are part of the "hematopoietic inductive microenvironment" (HIM), composed of marrow cells, i.e. macrophages, fibroblasts, adipocytes and endothelial cells. The Marrow cells maintain the functional integrity of the HIM by providing extra cellular matrix (ECM) proteins and basement membrane components that facilitate cell-cell contact. They also provide various soluble or resident cytokines needed for controlled hematopoietic cell differentiation and proliferation.

The interactions between the HSC and the stroma are required to preserve the viability of the HSCs and prevent their differentiation. Following HSCs transplantation, the transplanted HSCs must home into the bone marrow (BM) microenvironment and lodge in the appropriate niches before they proliferate and differentiate. During the homing process, the transplanted HSCs leave the bloodstream and transmigrate by following a gradient of chemokines across the

endothelial cell barrier of the BM to reach the dedicated niches. The donor HSCs must then home into the hematopoietic niches where they encounter a more favorable microenvironment for HSC division, and where, a continuum, physical and chemical contacts can be established between the HSCs and the mesenchymal cells, the ECM and the secreted growth factors. All these processes involve a complex array of molecules, such as cytokines, chemokines, hormones, steroids, extra cellular matrix proteins, growth factors, cell-to-cell interaction proteins, adhesion proteins, and matrix proteins.

The total number of cells engrafted in the BM dedicated niches underlies the success of HSCs transplant. To achieve engraftment, donor HSCs that are transplanted into the blood circulation should home into the recipient's marrow where they generate functional hematopoiesis foci. The number of these foci is concluded as the product of total HSCs transfused multiplied by their engraftment efficiency.

One of the major problems involved with HSC transplantation is the low survival rate of these cells in the acceptor system. It is well documented that HSC transplanted intravenously are cleared from the circulation and visualized in the BM within minutes after their transfusion. Three to five hours after HSCs transplantation, no donor cells are detected in the peripheral blood of the recipients [Askenasy et al 2002 Transplanted hematopoietic cells seed in clusters in recipient bone marrow in vivo. Stem Cells. 20:301-10]. The vast majority of the transplanted cells are destroyed shortly after being transfused. Consequently, the colonization of the recipient's marrow is of low efficiency and only 1-5 % of the transfused cells are detected in the recipient BM 2-3 days post transplantation [Kerre et al 2001 2001 Both CD34+38+ and CD34+38- cells home specifically to the bone marrow of NOD/LtSZ scid/scid mice but show different kinetics in expansion. J Immunol. 167:3692-8; Jetmore et al 2002 2002 Homing efficiency, cell cycle kinetics, and survival of quiescent and cycling human CD34(+) cells transplanted into conditioned NOD/SCID recipients. Blood. 99:1585-93].

Mesenchymal Stromal Cells (MSCs) are a heterogeneous population of cells, capable of differentiating into different types of mesenchymal mature cells. The differentiation of these cells to reticular endothelial cells, fibroblasts, adipocytes, and osteogenic precursor cells, depend upon influences from various bioactive factors.

The use of MSCs for the support of HSC engraftment is known in the art. Several publications have demonstrated higher engraftment efficiencies of HSC when

co-transplanted with mesenchymal stem cells [Gurevitch et al 1999 1999 Transplantation of allogeneic or xenogeneic bone marrow within the donor stromal microenvironment. Transplantation. 68:1362-8; Fan et al 2001 2001 Successful allogeneic bone marrow transplantation (BMT) by injection of bone marrow cells via portal vein: stromal cells as BMT-facilitating cells. [Stem Cells. 19:144-50]. It was also demonstrated that co-transplantation of human mesenchymal stem cells in a human-sheep engraftment model, resulted in the enhancement of long-term engraftment of human HSC chimeric BM in the animals [Almeida-Porada et al 2000] Co-transplantation of human stromal cell progenitors into preimmune fetal sheep results in early appearance of human donor cells in the circulation and boosts cell levels in bone marrow at later time points after transplantation [Blood. 95:3620-7]. It was found that simultaneous injection of HSC and mesenchymal stem cells accelerated hematopoiesis [Zhang et al 2004. Stem Cells 22:1256-62]. Recently, these finding were extended to a closer animal model – the Rhesus monkey. When haplo-identical HSC and mesenchymal stem cells were co-transplanted, facilitated HSC engraftment was demonstrated [Liu et al 2005 Zhonghua Xue Ye Xue Za Zhi. 26:385-8]. The use of mesenchymal stem cells to promote engraftment of HSC in human subjects was also recently reported [Koc ON, J Clin Oncol. 2000;18:307-316; Lazarus HM, Biol Blood Marrow Transplant. 2005 May;11(5):389-98].

Apparently the MSCs contribution to hematopoietic engraftment lies in the production of HSC supporting cytokines that help mediating and balancing the homing, self-renewal and commitment potentials of the transplanted HSCs, in rebuilding the damaged hematopoietic microenvironment needed for the homing and proliferation of the HSCs and in the inhibition of the donor derived T cells, which may cause Graft vs. Host Disease (GvHD), [Charbord P., and Moore, K., *Ann. N.Y. Acad. Sci.* 1044: 159–167 (2005); US patent nos. 6,010,696; 6555374]. For example, in a study by Maitra, [Maitra B, *et al.*, Bone Marrow Transplant. 33(6):597-604. (2004)], human mesenchymal stem cells were found to support unrelated donor hematopoietic stem cells and suppressed T-cell activation in NOD-SCID mice model, showing that unrelated, human bone marrow-derived MSCs may improve the outcome of allogeneic transplantation.

One major obstacle in using MSCs is the difficulty of isolating large quantities of normally occurring populations of these cells, which is technically difficult and costly, due in part, to the limited quantity of cells. The most obvious

source of MSCs is the bone marrow, but the significant discomfort involved in obtaining bone marrow aspirates and the risk of biopsy serve as drawbacks to these methods. The widely held belief that the human embryo and fetus constitute independent life makes the human embryo a problematic source of stem cells, adding a religious and ethical aspect to the already existing logistic difficulties.

Finding alternative sources for harvesting stem cells, has recently been attempted. Such alternative sources are for example adipose tissue, hair follicles, testicles, human olfactory mucosa, embryonic yolk sac, placenta, adolescent skin, and blood (e.g., umbilical cord blood and even menstrual blood). However, harvesting of stem cells from the alternative sources in adequate amounts for therapeutic and research purposes is still limited and generally laborious, involving, e.g., harvesting cells or tissues from a donor subject or patient, culturing and/or propagation of cells in vitro, dissection, etc.

The placenta is considered to be one of the most accessible sources of stem cells that does not involve any discomfort or ethical restraints. Placenta derived MSCs were found to have similar properties as BM derived MSC. They are plastic-adherent, express CD105, CD73 and CD90 membrane markers, and lack the expression of CD45, CD34, CD14, CD19 and HLA-DR surface molecules. However, unlike BM derived MSCs, placenta derived (PD)-MSCs treated with interferon- γ very minimally upregulated HLA-DR. Moreover, PD-MSCs cells exhibit immunosuppressive properties that are enhanced in the presence of interferon-γ. (Chang CJ, Yen ML, Chen YC, Chien CC, Huang HI, Bai CH, Yen BL. Placenta-derived Multipotent Cells exhibit immunosuppressive properties that are enhanced in the presence of interferon-gamma. Stem Cells. 2006 Nov;24(11):2466-77.

In addition to MSC markers PD-MSCs exhibit unique ESC surface markers of SSEA-4, TRA-1-61, and TRA-1-80, that suggest that these may be very primitive cells . (Yen BL, Huang HI, Chien CC, Jui HY, Ko BS, Yao M, Shun CT, Yen ML, Lee MC, Chen YC. Isolation of multipotent cells from human term placenta. Stem Cells. 2005;23(1):3-9). Moreover, PD-MSCs (Fetal origin), but not BM derived MSC are positive for the intracellular human leukocyte antigen-G (HLA). ? (Chang CJ, Yen ML, Chen YC, Chien CC, Huang HI, Bai CH, Yen BL. Placenta-derived multipotent cells exhibit immunosuppressive properties that are enhanced in the presence of interferon-gamma. Stem Cells. 2006 Nov;24(11):2466-77.)

Studies have shown that the expansion potential of PD- MSCs was significantly higher than that of adult BM-derived MSCs (Yen BL, Huang HI, Chien CC, Jui HY, Ko BS, Yao M, Shun CT, Yen ML, Lee MC, Chen YC. Isolation of Multipotent cells from Human Term Placenta. Stem Cells. 2005;23(1):3-9; M.J.S. de Groot-Swings, Frans H.J. Claas, Willem E. Fibbe and Humphrey H.H. Pieternella S. in 't Anker, Sicco A. Scherjon, Carin Kleijburg-van der Keur, Godelieve. Placenta Isolation of Mesenchymal Stem Cells of Fetal or Maternal Origin from Human. *Sem Cells,*2004;22;1338-1345.) In addition the placenta derived adherent cells can differentiate to osteoblasts, adipocytes and chondroblasts. Like BM derived MSCs, placenta derived MSCs were found to suppress umbilical cord blood (UCB) lymphocyte proliferation suggesting that combined transplantation of HSC and placenta derived (PD)-MSCs can reduce the potential graft-versus-host disease (GvHD) in recipients [Li CD, et al., Cell Res. Jul;15(7):539-47 (2005)], and can enhance hematopoietic support [ZhangYi et al., Chinese Medical Journal117(6): 882-887 (2004)]. The use of the placenta as a source for amniotic epithelial cells is taught for example in WO 00/73421, but obtaining these cells is still labor-intensive and the yield of the MSCs is very low.

Another way to solve the problem of the limited amount of MSCs is *ex-vivo* expansion of these cells using different culturing conditions [e.g. US Patent Nos. 6,326,198; 6030836; 6555374; 6,335,195; 6,338,942]. However, the drawback of such methods remains in the time-consuming, specific selection and isolation procedures they require, rendering these methods costly and fastidious.

Three dimensional (3D) culturing of cells was found in several studies to be more effective in yield [Ma T, et al., Biotechnology Progress. Biotechnol Prog 15:715-24 (1999); Yubing Xie, Tissue Engineering 7(5): 585-598 (2001)]. The Use of 3D culturing procedures which mimic the natural environment of the MSCs is based on seeding these cells in a perfusion bioreactor containing Polyactive foams [Wendt, D. et al., Biotechnol Bioeng 84: 205-214, (2003)] tubular poly-L-lactic acid (PLLA) porous scaffolds in a Radial-flow perfusion bioreactor [Kitagawa et al., Biotechnology and Bioengineering 93(5): 947-954 (2006)], and a plug flow bioreactor for the growth and expansion of hematopoietic stem cells (U.S. Patent No. 6,911,201).

A three-dimensional framework, which attaches stromal cells, was suggested in U.S. Pat. No. 6,022,743, and sponge collagen was suggested as a 3D matrix in Hosseinkhani, H et al., [Tissue Engineering 11(9-10): 1476-1488 (2005)]. However,

the use of MSCs, grown in these conditions for supporting *in vivo* engraftment of HSCs following HSC transplantation has never been suggested in any of these studies. Also, time consuming optimization of various conditions e.g., perfusion conditions, or various isolation techniques for specific cell types were required.

The use of a perfused Post-partum placenta as a 3D reactor for culturing MSCs was suggested in US Pat. No. 7045148 and U.S. Pat. App. Nos. 20020123141 20030032179 and 2005011871. However, this procedure is limited for up to 24 hours after the placenta is isolated and involves perfusion, therefore mass growth of the cells and its maintenance for prolonged time periods is not possible.

There is thus a widely recognized need for, and it would be highly advantageous to have, novel methods of cell expansion and uses of cells and conditioned medium produced thereby for therapy and which are devoid of the above limitations.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a method of cell expansion, the method comprising culturing adherent cells from placenta or adipose tissue under three-dimensional culturing conditions, which support cell expansion.

According to another aspect of the present invention there is provided a method of producing a conditioned medium, the method comprising: culturing adherent cells from a placenta or adipose tissue in three dimensional culturing conditions which allow cell expansion; and collecting a conditioned medium of the expanded adherent cells, thereby producing the conditioned medium.

According to yet another aspect of the present invention there is provided a population of cells generated according to the method as above.

According to still another aspect of the present invention there is provided an isolated population of cells comprising adherent cells of placenta or adipose tissue, wherein the adherent cells secrete a higher level of at least one factor selected from the group consisting of SCF, IL-6, and Flt-3 than that secreted by adherent cells of placenta or adipose tissue grown in a 2D culture.

According to an additional aspect of the present invention there is provided an isolated population of cells comprising adherent cells of placenta or adipose tissue, wherein the adherent cells express a higher level of at least one protein selected from

the group consisting of H2A histone family (H2AF), Aldehyde dehydrogenase X (ALDH X), eukaryotic translation elongation factor 2 (EEEF2), reticulocalbin 3, EF-hand calcium binding domain (RCN2) and calponin 1 basic smooth muscle (CNN1) than that expressed by adherent cells of placenta or adipose tissue grown in a 2D culture.

According to yet an additional aspect of the present invention there is provided an isolated population of cells comprising adherent cells of placenta or adipose tissue, wherein the adherent cells express a lower level of expression of at least one protein selected from the group consisting of heterogeneous nuclear ribonucleoprotein H1 (Hnrph1), CD44 antigen isoform 2 precursor, 3 phosphoadenosine 5 phosphosulfate synthase 2 isoform a (Papss2) and ribosomal protein L7a (rpL7a) than that expressed by adherent cells of placenta or adipose tissue grown in a 2D culture.

According to still an additional aspect of the present invention there is provided an isolated population of cells comprising adherent cells of placenta or adipose tissue, wherein the adherent cells are characterized by a higher immunosuppressive activity than that of adherent cells of placenta or adipose tissue grown in a 2D culture.

According to further features in preferred embodiments of the invention described below the immunosuppressive activity comprises reduction in T cell proliferation.

According to further aspect of the present invention there is provided a pharmaceutical composition comprising, as an active ingredient, the population of cells generated according to the method as above.

According to a further aspect of the present invention there is provided a pharmaceutical composition comprising, as an active ingredient, the conditioned medium produced according to the method as above.

According to yet a further aspect of the present invention there is provided a pharmaceutical composition comprising, as an active ingredient, the isolated population of cells according to above.

According to still a further aspect of the present invention there is provided a method of treating a condition which may benefit from stromal cell transplantation 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 placenta and adipose tissue, thereby treating the condition which may benefit from stem cell transplantation in the subject.

According to still a further aspect of the present invention there is provided a method of treating a condition which may benefit from stromal cell transplantation in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a conditioned medium of adherent cells derived from a tissue selected from the group consisting of placenta and adipose tissue, thereby treating the condition which may benefit from stem cell transplantation in the subject.

According to still a further aspect of the present invention there is provided a method of reducing an immune response in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the isolated population of cells of claims 3, 4, 5, 6 or 7, so as to reduce the immune response in the subject.

According to still further features in the described preferred embodiments the subject is treated with cell therapy.

According to still further features in the described preferred embodiments the method further comprises administering stem cells.

According to still further features in the described preferred embodiments the stem cells comprise hematopoietic stem cells.

According to still further features in the described preferred embodiments the cells are administered concomitantly with the conditioned medium or adherent cells.

According to still further features in the described preferred embodiments the cells are administered following administration of the conditioned medium or adherent cells.

According to still further features in the described preferred embodiments the adherent cells are obtained from a three dimensional culture.

According to still further features in the described preferred embodiments the adherent cells are obtained from a two dimensional culture.

According to still further features in the described preferred embodiments the condition is selected from the group consisting of stem cell deficiency, heart disease, Parkinson's disease, cancer, Alzheimer's disease, stroke, burns, loss of tissue, loss of blood, anemia, autoimmune disorders, diabetes, arthritis, Multiple Sclerosis, graft vs. host disease (GvHD), neurodegenerative disorders, autoimmune encephalomyelitis

(EAE), systemic lupus erythematosus (SLE), rheumatoid arthritis, systemic sclerosis, Sjorgen's syndrome, multiple sclerosis (MS), Myasthenia Gravis (MG), Guillain-Barré Syndrome (GBS), Hashimoto's Thyroiditis (HT), Graves's Disease, Insulin dependent Diabetes Melitus (IDDM) and Inflammatory Bowel Disease.

According to still further features in the described preferred embodiments the three dimensional culture comprises a 3D bioreactor.

According to still further features in the described preferred embodiments the bioreactor is selected from the group consisting of a plug flow bioreactor, a continuous stirred tank bioreactor and a stationary-bed bioreactor.

According to still further features in the described preferred embodiments the culturing of the cells is effected under a continuous flow of a culture medium.

According to still further features in the described preferred embodiments the three dimensional culture comprises an adherent material selected from the group consisting of a polyester, a polyalkylene, a polyfluorochloroethylene, a polyvinyl chloride, a polystyrene, a polysulfone, a cellulose acetate, a glass fiber, a ceramic particle, a matrigel, an extracellular matrix component, a collagen, a poly L lactic acid and an inert metal fiber.

According to still further features in the described preferred embodiments the culturing is effected for at least 3 days.

According to still further features in the described preferred embodiments the culturing is effected for at least 3 days.

According to still further features in the described preferred embodiments the culturing is effected until the adherent cells reach at least 60 % confluence.

According to still further features in the described preferred embodiments the condition may benefit from the facilitation of hematopoietic stem cell engraftment.

According to still further features in the described preferred embodiments the adherent cells comprise a positive marker expression array selected from the group consisting of CD73, CD90, CD29 and CD105.

According to still further features in the described preferred embodiments the adherent cells comprise a negative marker expression array selected from the group consisting of CD45, CD80, HLA-DR, CD11b, CD14, CD19, CD34 and CD79.

According to still further features in the described preferred embodiments the adherent cells secrete a higher level of at least one factor selected from the group

consisting of SCF, Flt-3 and IL-6 higher than that secreted by adherent cells from placenta or adipose tissue grown in a 2D culture.

According to still further features in the described preferred embodiments the adherent cells express a higher level of at least one protein selected from the group consisting of H2A histone family (H2AF), Aldehyde dehydrogenase X (ALDH X), eukaryotic translation elongation factor 2 (EEEF2), reticulocalbin 3, EF-hand calcium binding domain (RCN2) and calponin 1 basic smooth muscle (CNN1) than that secreted by adherent cells from placenta or adipose tissue grown in a 2D culture.

According to still further features in the described preferred embodiments the adherent cells express a lower level of expression of at least one protein selected from the group consisting of heterogeneous nuclear ribonucleoprotein H1 (Hnrph1), CD44 antigen isoform 2 precursor, 3 phosphoadenosine 5 phosphosulfate synthase 2 isoform a (Papss2) and ribosomal protein L7a (rpL7a) than that secreted by adherent cells from placenta or adipose tissue grown in a 2D culture.

According to still further features in the described preferred embodiments the adherent cells or medium are characterized by a higher immunosuppressive activity than that of adherent cells of placenta or adipose tissue grown in a 2D culture.

According to still further features in the described preferred embodiments the immunosuppressive activity comprises reduction in T cell proliferation.

According to still further features in the described preferred embodiments the cells comprise cells having a stromal stem cell phenotype.

According to still further features in the described preferred embodiments the stromal stem cell phenotype comprises T cell suppression activity.

According to still further features in the described preferred embodiments thstromal stem cell phenotype comprises hematopoietic stem cell support activity.

According to still further features in the described preferred embodiments the use of the population of cells described above is for manufacture of a medicament identified for transplantation.

The present invention successfully addresses the shortcomings of the presently known configurations by providing novel methods of cell expansion and uses of cells and conditioned medium produced thereby for therapy.

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 present 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.

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 preferred embodiments of the present 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 depicts 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 Stromal Cells (3D-ASC), imitating the bone micro-environment (Figure 1b). Figures 1c-f are electron micrographs depicting the PluriX™ 3D matrix seeded with 3D-ASC, 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 stromal cells (3D-ASC; Lots 5-8) originating from placenta, grown in 3D growth conditions within the bioreactor systems. ASCs (2×10^6) were seeded in the bioreactor at a density of

10000 - 15000 cells / a carrier. Following a 12 day culture 3D-ASCs 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-ASC (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 MSCs (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). ASCs 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 ASCs 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 ASCs. 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 ASCs 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-ASC to osteoblasts. Human placenta derived ASC 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) 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 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-ASC; 2 mice, b), or placenta derived adherent cells cultured in 3D conditions (3D-ASC), in the pluriX™ 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 co-transplanted with 2D-ASC (b) as well as with 3D-ASC (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-ASC cultured cells in comparison to mice treated with 2D-ASC cultured cells indicates a higher therapeutic advantage unique to 3D cultured ASCs.

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 ASCs. (Figure 7b). Note the significantly higher percentage of human hematopoietic population (hCD45+) (7a – 29 %) in a mouse co-transplanted with adipose tissue derived ASC in comparison to a mouse treated with human CD34+ alone (7b -12 %).

FIG. 8 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 ASCs, or a combination of PBMC and 2D and 3D cultured placental ASCs (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.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is of novel methods of cell expansion and uses of cells and conditioned medium produced thereby, for stem cell related therapy, stem cell engraftment and HSC support.

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

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not 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.

In the developing medical world, there is a growing need for stem cells, and more specifically for stromal stem cells (also termed "mesenchymal stem cells"), for clinical and research purposes. MSCs are used for support of HSC transplantation and engraftment and also for curing a growing number of conditions e.g., heart diseases, BM deficiencies, neuronal related diseases, and conditions which require organ or tissue transplantation.

Obstacles in using stem cells lie in the technical difficulty of isolating large quantities of normally occurring populations of stem or progenitor cells, due to limited quantity of these cells in most tissues, the discomfort and risk involved in the procedures for obtaining stem cells, and the accompanying loss of memory B cells and hematopoietic stem cells with present harvesting procedures. Obtaining cells from the human embryo add a religious and ethical aspect to the already existing technical difficulties.

Alternative sources for bone marrow-derived stem cells include adipose tissues and placenta. However, currently there are no methods for efficient expansion of stem cells from such tissues.

While reducing the present invention to practice, the present inventors have uncovered that adherent cells from placenta or adipose tissue can be efficiently propagated in 3D culturing conditions. Surprisingly, the present inventors uncovered

that such cells comprise functional properties which are similar to those of MSCs and therefore these cells and the conditioned medium produced there from, can be used for therapeutic purposes such as transplantation, tissue regeneration and *in vivo* HSC support.

As is illustrated herein below and in the Examples section which follows, the present inventors were able to expand adipose and placenta-derived adherent cells which comprise stromal stem cells properties in 3D settings. 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 and (see Figures 3a-b). Most importantly, adipose and placenta derived adherent cells propagated on 2D or 3D settings were able to support HSC engraftment (see Example 2), substantiating the use of the cells of the present invention, as stromal stem cells, in the clinic.

Thus, according to one aspect of the present invention, there is provided a method of cell expansion.

The method comprising culturing adherent cells from placenta or adipose tissue under three-dimensional (3D) culturing conditions which support cell expansion.

As used herein the terms "expanding" and "expansion" refer to substantially differentiationless 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 differentiationless cell renewal, i.e., substantially stationary cell population without differentiation accompanying such stationarity.

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).

As used herein the phrase "three dimensional culturing conditions" refers to disposing the cells 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) as 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 three dimensional culturing conditions of the present invention are designed to mimic such as environment as is further exemplified below.

Thus, adherent cells of this aspect of the present invention are retrieved from an adipose or placental tissue.

Placental cells may be obtained from a full-term or pre-term placenta. Placenta are 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; most preferably the plancental tissue is human. A convenient source of plancental tissue is from a post partum placenta (e.g., 1-6 hours), however, the source of plancental 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 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.

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. A preferred source of adipose tissue is omental adipose. In humans, the adipose is typically isolated by liposuction.

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 between 20 microns to 800 microns. 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 present invention 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, 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 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. This may be effected prior to (see Example 1) or concomitant with culturing in 3D culturing conditions.

As used herein "an adherent material" refers to a synthetic, naturaly 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 present invention include, but are not limited to, a polyester, 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 present 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 present 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, cytokines, 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 pigogram/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).

Once adherent cells are at hand they may be passaged to three dimensional settings (see Example 1 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 (as mentioned hereinabove).

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

For example, for a growth matrix of 0.5 mm in height, the increase is by a factor of at least from 5 to 30 times, calculated by projection onto a base of the growth matrix. Such an increase by a factor of about 5 to 30 times, is per unit layer, and if a plurality of such layers, either stacked or separated by spacers or the like, is used, the factor of 5 to 30 times applies per each such structure. When the matrix is used in sheet form, preferably non-woven fiber sheets, or sheets of open-pore foamed polymers, the preferred thickness of the sheet is about 50 to 1000 μm or more, there being provided adequate porosity for cell entrance, entrance of nutrients and for removal of waste products from the sheet. According to a preferred embodiment the pores have an effective diameter of 10 μm to 100 μm. Such sheets can be prepared from fibers of various thicknesses, the preferred fiber thickness or fiber diameter range being from about 0.5 μm to 20 μm, still more preferred fibers are in the range of 10 μm to 15 μm in diameter.

The structures of the invention may be supported by, or even better bonded to, a porous support sheet or screen providing for dimensional stability and physical strength.

Such matrix sheets may also be cut, punched, or shredded to provide particles with projected area of the order of about 0.2 mm^2 to about 10 mm^2, with the same order of thickness (about 50 to 1000 μm).

Further details relating to the fabrication, use and/or advantages of the growth matrix which was used to reduce the present invention to practice are described in U.S. Pat. Nos. 5,168,085, and in particular, 5,266,476, both are incorporated herein by reference.

The adherent surface may have a shape selected from the group consisting of squares, rings, discs, and cruciforms.

For high scale production, culturing is preferably effected in a 3D bioreactor.

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

As shown Example 1 of the Examples section, a three dimensional (3D) plug flow bioreactor (as described in US Pat. No. 6911201) is capable of supporting the growth and prolonged maintenance of stromal cells. In this bioreactor, stromal cells are seeded on porrosive carriers made of a non woven fabric matrix of polyester, packed in a glass column, thereby enabling the propagation of large cell numbers in a relatively small volume.

The matrix used in the plug flow bioreactor can be of sheet form, non-woven fiber sheets, or sheets of open-pore foamed polymers, the preferred thickness of the sheet is about 50 to 1000 μm or more, there being provided adequate porosity for cell entrance, entrance of nutrients and for removal of waste products from the sheet.

Other 3D bioreactors that can be used with the present invention include, but are not 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 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). Other bioreactors which can be used in accordance with the present 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.

Cells are preferably harvested once reaching at least about 40 % confluence, 60 % confluence or 80 % confluence while preferably avoiding uncontrolled differentiation and senescence.

Culturing is effected for at least about 2 days, 3 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. Passaging may also be effected to increase cell number.

Adherent cells of the present invention preferably 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+, 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.

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 adipogenic, hepatogenic, osteogenic and neurogenic differentiation.

Any of these structural or functional features can be used to qualify the cells of the present invention (see Examples 1-2 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, H2AF or ALDH X at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or preferably 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 present invention secrete or express IL-6, EEEF2, RCN2 or 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 present 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 Hnrph1, CD44 antigen isoform 2 precursor, Papss2 or rpL7a expressed or secreted by adherent cells of placenta or adipose tissue grown in a 2D culture.

While further reducing the present invention to practice the present inventors have realized that adherent stromal cells, and particularly 3D-ASCs, showed immunosuppressive activity. As is shown in Example 3 of the Examples section which follows, Adherent stromal cells, and particularly 3D-ASCs, were found to suppress the immune reaction of human cord blood mononuclear cells in an MLR assay. Thus, the cells of the present invention may comprise biological activities which may be preferentially used in the clinic (e.g., T cell suppression activity, hematopoietic stem cell support activity).

While further reducing the present invention to practice the present inventors have realized that conditioned medium of the cells of the present invention may comprise biological activities which may be preferentially used in the clinic (e.g., T cell suppression activity, hematopoietic stem cell support activity).

Thus, the present invention further envisages collection of conditioned medium and its use as is or following further steps of concentration, enrichment or fractionation using methods which are well known in the art. Preferably a conditioned medium of the present is obtained from a high viability mid-log culture of cells.

As mentioned hereinabove, cells and conditioned media of the present invention are characterized by a stromal stem cell phenotype and as such can be used in any research and clinical application which may benefit from the use of such cells.

Engraftment and initiation of hematopoiesis by transplanted HSCs depend on complex processes which include homing, following a gradient of chemokines across

the endothelial cell barrier, to the bone marrow and lodging in the appropriate niches, while establishing physical contacts between transplanted cells, the ECM and the mesenchymal cells of the niches. All these processes involve a complex array of molecules, such as cytokines, hormones, steroids, extra cellular matrix proteins, growth factors, cell-to-cell interaction and adhesion proteins, and matrix proteins.

It is known that only 1-5 % of transfused HSCs are detected in the recipient BM 2-3 days post transplantation [Kerre et al., J Immunol. 167:3692-8. (2001); Jetmore et al., Blood. 99:1585-93 (2002)].

MSCs contribution to hematopoietic engraftment is in part by the inhibition of donor derived T cell production, which cause graft vs. host disease [GvHD, Charbord P., and Moore, K., *Ann. N.Y. Acad. Sci.* 1044: 159–167 (2005); Maitra B, et al., Bone Marrow Transplant. 33(6):597-604. (2004); US patent nos. 6,010,696; 6555374]; and part by providing a hematopoietic stem cell (HSC) support (i.e., sustaining and aiding the proliferation, maturation and/or homing of hematopoietic stem cells).

As shown in Example 2 of the Examples section which follows, placenta and adipose tissue-derived adherent cells were surprisingly found to be supportive of HSC engraftment even after chemotherapy.

Given these results it is conceivable that cells or media of the present invention may be used in any clinical application for which stromal stem cell transplantation is used.

Thus, according to another aspect of the present invention there is provided a method of treating a medical condition (e.g., pathology, disease, syndrome) which may benefit from stromal stem cell transplantation in a subject in need thereof.

As used herein the term "treating" refers to inhibiting or arresting the development of a pathology and/or causing the reduction, remission, or regression of a pathology. Those of skill in the art will understand that various methodologies and assays can be used to assess the development of a pathology, and similarly, various methodologies and assays may be used to assess the reduction, remission or regression of a pathology. Preferably, the term "treating" refers to alleviating or diminishing a symptom associated with a cancerous disease. Preferably, treating cures, e.g., substantially eliminates, the symptoms associated with the medical condition.

As used herein "a medical condition which may benefit from stromal stem cell transplantation" refers to any medical condition which may be alleviated by administration of cells/media of the present invention.

The term or phrase "transplantation", "cell replacement" or "grafting" are used interchangeably herein and refer to the introduction of the cells of the present invention to target tissue.

As used herein the term "subject" refers to any subject (e.g., mammal), preferably a human subject.

The method of this aspect of the present invention comprises administering to the subject a therapeutically effective amount of the cells or media of the present invention (described hereinabove), thereby treating the medical condition which may benefit from stromal stem cell transplantation in the subject

Cells which may be administered in accordance with this aspect of the present invention include the above-described adherent cells which may be cultured in either two-dimensional or three-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 present invention are well known in the art. See for example, U.S. Pat. Nos. 5,486,359, 5,942,225, 5,736,396, 5,908,784 and 5,902,741.

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

The cells and media may be of autologous or non-autologous source (i.e., allogenic or xenogenic) of fresh or frozen (e.g., cryo-preserved) preparations.

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

Thus, for example, for improving stem cell engraftment (e.g., increasing the number of viable HSC in the recipient BM and optimally improve normal white blood cell count) the cells/media of the present invention may be administered prior to, concomitantly with or following HSC transplantation.

Preferably the HSCs and stromal cells share common HLA antigens. Preferably, the HSCs and stromal cells are from a single individual. Alternatively, the HSCs and stromal cells are from different individuals.

The term or phrase "transplantation", "cell replacement" or "grafting" are used interchangeably herein and refer to the introduction of the cells of the present

invention to target tissue. The cells can be derived from the recipient or from an allogeneic or xenogeneic donor.

Since non-autologous cells are likely to 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, semipermeable membranes before transplantation.

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

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

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

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

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

Examples of immunosuppressive agents include, but are not limited to, methotrexate, cyclophosphamide, cyclosporine, cyclosporin A, chloroquine, hydroxychloroquine, sulfasalazine (sulphasalazopyrine), gold salts, D-penicillamine, leflunomide, azathioprine, anakinra, infliximab (REMICADE), etanercept, 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 or media 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 one or more of the chemical conjugates described herein, with other chemical components such as pharmaceutically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound 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 present 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 present 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 present 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, or physiological salt buffer. 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.

Following transplantation, the cells of the present invention preferably survive in the diseased area for a period of time (e.g. at least 6 months), such that a therapeutic effect is observed.

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

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

EXAMPLES

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

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological 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 Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

EXAMPLE 1

Production and culturing of adherent stromal cells (ASC) from bone marrow, placenta and adipose tissues

Adherent cells were cultured in a bioreactor system containing 3D carriers to produce 3D-ASC cells, characterized by a specific cell marker expression profile. 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 stromal cells – Bone marrow (BM) stromal cells were obtained from aspirated sterna marrow of hematologically healthy donors undergoing open-heart 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/cm3) were collected, washed 3 times in HBSS and resuspended in growth media [DMEM

(Biological Industries, Beit 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 ug/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 bioreactors.

Placenta derived stromal 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 h at 37 °C with 0.1 % Collagenase (1mg / ml tissue; Sigma- Aldrich, St. Lewis, MO). Using gentle pipeting, suspended cells were then washed with DMEM supplemented with 10 % FCS, Pen-Strep-Nystatin mixture (100 U/ml:100 ug/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 % 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 stromal cells – Stromal cells were obtained from human adipose tissue of liposuction procedures (Rambam Haifa, Israel). Adipose tissue was washed extensively with equal volumes of PBS and digested at 37 °C for 30 min with collagenase (20 mg/ml). Cells were then washed with DMEM containing 10 % FCS, Pen-Strep-Nystatin mixture (100 U/ml:100 ug/ml:1.25 un/ml) and L- Glutamin and centrifuged at 1200 rpm for 10 min RT, resuspended with lysing solution (1:10; Biological Industries, Beit Ha'emek, Israel, in order to discard red-blood cells) centrifuged and resuspended with DMEM containing 10 % FCS, Pen-Strep-Nystatin mixture (100 U/ml:100 ug/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 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 % confluece, 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), 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. The bioreactor was maintained in an incubator of 37 °C, with flow rate regulated and monitored by a valve (6a in Figure 1g), and 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 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 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 stromal cells (3D-ASC) - Non-confluent primary human adherent 2D cell cultures, grown as described above, were trypsinized, washed, resuspended in DMEM supplemented with 10 % FBS, Pen-Strep-Nystatin mixture (100 U/ml:100 ug/ml:1.25 un/ml) and 2 mM L-glutamine, and seeded (10^3-10^5 cells/ml) via an injection point onto the 3D carriers in a sterile Plug Flow bioreactor (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 mixture (100 U/ml:100 ug/ml:1.25 un/ml). Flow was kept at a rate of 0.1-5 ml/min. Seeding process involved cease of circulation for 2- 48 hrs, thereby allowing the cells to settle on the carriers. Bioreactor was kept under controlled temperature (37 °C) and pH conditions (pH = 6.7-7.4); using an incubator supplied with sterile air and CO_2 as needed. Growth medium was replaced 2-3 times

a week. Circulation medium was replaced with fresh DMEM media, every 4 hr to 7 days. At a density of 1 X 10^6-1 X 10^7 cells/ml (following 12-40 days of growth), total medium volume was removed from the bioreactor and bioreactor and carriers were washed 3-5 times with PBS. 3D-ASC 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-ASC quality biological assays – Cryopreserved 3D-ASC cells 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-ASC membrane marker phenotype was analyzed using fluorescence monoclonal antibodies flow-cytometer (Beckman Coulter, Fullerton, CA).

Comparison between the cell membrane marker profile of 3D and 2D cultured adherent cells using flow cytometery 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, 30min, dark conditions) with saturating concentrations of each of the following MAbs: FITC-conjugated anti-human CD90 (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 (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).

Comparison between the protein profile of 3D and 2D cultured adherent cells using mass spectrometry analysis - 2D and 3D derived culturing procedures ASCs 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 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. Breifly, iTRAQ reagents are non-polymeric, isobaric tagging reagents. Peptides within each sample are labeled with one of four isobaric, isotope-coded tags via their N-terminal and/or lysine side chains. The four labeled samples are mixed and peptides are analyzed with mass spectrometery. 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: http://docs.appliedbiosystems.com/pebiodocs/00113379.pdf).

Proteomics analysis of 2D culture versus 3D culture of placenta derived ASCs 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 analyzed were: heterogeneous nuclear ribonucleoprotein H1 (Hnrph1 GeneBank Accession No. NP_005511), H2A histone family (H2AF, GeneBank Accession No. NP_034566.1), eukaryotic translation elongation factor 2 (EEEF2, GeneBank Accession No. NP_031933.1), reticulocalbin 3, EF-hand calcium binding domain (RCN2, GeneBank Accession No. NP_065701), CD44 antigen isoform 2 precursor (GeneBank Accession No. NP_001001389, calponin 1 basic smooth muscle (CNN1, GeneBank Accession No. NP_001290), 3 phosphoadenosine 5 phosphosulfate synthase 2 isoform a (Papss2, GeneBank Accession No. NP_004661), ribosomal protein L7a (rpL7a, GeneBank Accession No. NP_000963) and Aldehyde dehydrogenase X (ALDH X, GeneBank Accession No. P47738). Every experiment was done twice. Because of the 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 ASCs 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, Trombopoietin (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 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).

RESULTS

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 seeding in Figure 1b). As is shown in Figures 1c-f, bone marrow produced 3D-ASC 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 - Different production lots of placenta derived 3D-ASC 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 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 successful transplantation.

3D-ASCs 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 that 3D-ASCs 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. ASCs of both 2D and 3D placenta cultures, did not express any hematopoietic membrane markers (Figure 3b).

3D-ASCs 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 ASCs, the profile of the four main hematopoietic secreted proteins in the conditioned media of 2D and 3D ASC 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 equal in both cultures.

3D-ASCs show a unique protein profile in mass spectrometry analysis – In order to further define the difference between 2D and 3D cultured ASCs, the protein profile of these cells was analyzed by mass spectrometry. Figure 4d shows that 2D and 3D cultured ASCs show a remarkably different protein expression 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)			
	3D cultured ASCs		2D cultured ASCs	
	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-ASCs have the capacity to differentiate into osteoblasts - In order to further characterize 3D-ASCs, 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-ASC have the capacity to differentiate *in vitro* to osteoblasts cells.

EXAMPLE 2

Assessment of the Ability of placenta derived 3D-ASC to improve HSC engraftment

3D-ASC 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 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 post irradiation) transplanted with 50,000-100,000 $hCD34^+$ cells, with or without additional ASCs (0.5×10^6 - 1×10^6) 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 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 %.

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 10^6 ASCs, 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.

RESULTS

3D-ASC improved engraftment of HSC in irradiated mice - Human CD34+ hematopoietic cells and 3D-ASC derived from placenta or adipose 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 and Figure 6, co-transplantation of 3D-ASC 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-ASC from placenta	5.1	12.2
CD34+3D-ASC from adipose	8.7	9.6

3D-ASC improved engraftment of HSC in mice treated with chemotherapy - Human CD34+ hematopoietic cells were co-transplanted with 500,000- 2D-ASC or 3D-ASC 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, co-transplantation of ASC 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-ASC) than in the mice co-transplantation 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-ASC from placenta	6.0	7.9

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

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

EXAMPLE 3

The suppression of lymphocyte response by 2D and 3D cultured ASCs

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

MATERIALS AND EXPERIMENTAL PROCEDURES

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

incompatible lymphocytes in mixed culturing of responsive (proliferating) and stimulating (unproliferative) cells. Human cord blood (CB) mononuclear cells (2 X 10^5) were used as responsive cells and were stimulated by being co-cultured with equal amounts (10^5) of irradiated (3000Rad) human peripheral blood derived Monocytes (PBMC), or with 2D or 3D cultured adherent cells, produced from the placenta, or a 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 1μC ^{3}H-thymidine during the last 18 hr of culturing. Cells were then harvested over fiberglass filter and thymidine uptake was quantified with a scintillation counter.

RESULTS

Figure 8 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 present 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, ASCs 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-ASCs described hereinabove, the 3D ASCs were more immunosuppressive.

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 present invention.

WHAT IS CLAIMED IS:

1. A method of cell expansion, the method comprising culturing adherent cells from placenta or adipose tissue under three-dimensional culturing conditions, which support cell expansion.

2. A method of producing a conditioned medium, the method comprising

(a) culturing adherent cells from a placenta or adipose tissue in three dimensional culturing conditions which allow cell expansion; and

(b) collecting a conditioned medium of said expanded adherent cells, thereby producing the conditioned medium.

3. A population of cells generated according to the method of claim 1.

4. An isolated population of cells comprising adherent cells of placenta or adipose tissue, wherein said adherent cells secrete a higher level of at least one factor selected from the group consisting of SCF, IL-6, and Flt-3 than that secreted by adherent cells of placenta or adipose tissue grown in a 2D culture.

5. An isolated population of cells comprising adherent cells of placenta or adipose tissue, wherein said adherent cells express a higher level of at least one protein selected from the group consisting of H2A histone family (H2AF), Aldehyde dehydrogenase X (ALDH X), eukaryotic translation elongation factor 2 (EEEF2), reticulocalbin 3, EF-hand calcium binding domain (RCN2) and calponin 1 basic smooth muscle (CNN1) than that expressed by adherent cells of placenta or adipose tissue grown in a 2D culture.

6. An isolated population of cells comprising adherent cells of placenta or adipose tissue, wherein said adherent cells express a lower level of expression of at least one protein selected from the group consisting of heterogeneous nuclear ribonucleoprotein H1 (Hnrph1), CD44 antigen isoform 2 precursor, 3 phosphoadenosine 5 phosphosulfate synthase 2 isoform a (Papss2) and ribosomal protein L7a (rpL7a) than that expressed by adherent cells of placenta or adipose tissue grown in a 2D culture.

7. An isolated population of cells comprising adherent cells of placenta or adipose tissue, wherein said adherent cells are characterized by a higher immunosuppressive activity than that of adherent cells of placenta or adipose tissue grown in a 2D culture.

8. The isolated population of cells of claim 7, wherein said immunosuppressive activity comprises reduction in T cell proliferation.

9. A pharmaceutical composition comprising, as an active ingredient, the population of cells generated according to claim 1.

10. A pharmaceutical composition comprising, as an active ingredient, the conditioned medium produced according to claim 2.

11. A pharmaceutical composition comprising, as an active ingredient, the isolated population of cells of claims 4, 5, 6 or 7.

12. A method of treating a condition which may benefit from stromal cell transplantation 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 placenta and adipose tissue, thereby treating the condition which may benefit from stem cell transplantation in the subject.

13. A method of treating a condition which may benefit from stromal cell transplantation in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of a conditioned medium of adherent cells derived from a tissue selected from the group consisting of placenta and adipose tissue, thereby treating the condition which may benefit from stem cell transplantation in the subject.

14. A method of reducing an immune response in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of the isolated population of cells of claims 3, 4, 5, 6 or 7, so as to reduce the immune response in the subject.

15. The method of claim 14, wherein the subject is treated with cell therapy.

16. The method of claim 12 or 13, further comprising administering stem cells.

17. The method of claim 16, wherein said stem cells comprise hematopoietic stem cells.

18. The method of claim 16, wherein said stem cells are administered concomitantly with said conditioned medium or adherent cells.

19. The method of claim 16, wherein said stem cells are administered following administration of said conditioned medium or adherent cells.

20. The method of claims 12 and 13, wherein said adherent cells are obtained from a three dimensional culture.

21. The method of claims 12 and 13, wherein said adherent cells are obtained from a two dimensional culture.

22. The method of claims 12 and 13, wherein said condition is selected from the group consisting of stem cell deficiency, heart disease, Parkinson's disease, cancer, Alzheimer's disease, stroke, burns, loss of tissue, loss of blood, anemia, autoimmune disorders, diabetes, arthritis, Multiple Sclerosis, graft vs. host disease (GvHD), neurodegenerative disorders, autoimmune encephalomyelitis (EAE), systemic lupus erythematosus (SLE), rheumatoid arthritis, systemic sclerosis, Sjorgen's syndrome, multiple sclerosis (MS), Myasthenia Gravis (MG), Guillain-Barré Syndrome (GBS), Hashimoto's Thyroiditis (HT), Graves's Disease, Insulin dependent Diabetes Melitus (IDDM) and Inflammatory Bowel Disease.

23. The method or cell population of any of claims 1, 2, 3 or 20, wherein said three dimensional culture comprises a 3D bioreactor.

24. The method of claim 23, wherein said bioreactor is selected from the group consisting of a plug flow bioreactor, a continuous stirred tank bioreactor and a stationary-bed bioreactor.

25. The method or cell population of any of claims 1, 2, 3 or 20, wherein said culturing of said cells is effected under a continuous flow of a culture medium.

26. The method or cell population of any of claims 1, 2, 3 or 20, wherein said three dimensional culture comprises an adherent material selected from the group consisting of a polyester, a polyalkylene, a polyfluorochloroethylene, a polyvinyl chloride, a polystyrene, a polysulfone, a cellulose acetate, a glass fiber, a ceramic particle, a matrigel, an extracellular matrix component, a collagen, a poly L lactic acid and an inert metal fiber.

27. The method or cell population of any of claims 1, 2, 3, or 20, wherein said culturing is effected for at least 3 days.

28. The method or cell population of claim 21, wherein said culturing is effected for at least 3 days.

29. The method or cell population of any of claims 1, 2, 3 or 20, wherein said culturing is effected until said adherent cells reach at least 60 % confluence.

30. The methods of claims 12 and 13, wherein the condition may benefit from the facilitation of hematopoietic stem cell engraftment.

31. The methods, population of cells or medium of claims 1, 2, 3, 4, 5, 6, 7 or 20, wherein said adherent cells comprise a positive marker expression array selected from the group consisting of CD73, CD90, CD29 and CD105.

32. The methods, population of cells or medium of claims 1, 2, 3, 4, 5, 6, 7 or 20, wherein said adherent cells comprise a negative marker expression array selected from the group consisting of CD45, CD80, HLA-DR, CD11b, CD14, CD19, CD34 and CD79.

33. The methods, population of cells or medium of claims 1, 2, 3, or 20, wherein said adherent cells secrete a higher level of at least one factor selected from the group consisting of SCF, Flt-3 and IL-6 higher than that secreted by adherent cells from placenta or adipose tissue grown in a 2D culture.

34. The methods, population of cells or medium of claims 1, 2, 3, or 20, wherein said adherent cells express a higher level of at least one protein selected from the group consisting of H2A histone family (H2AF), Aldehyde dehydrogenase X (ALDH X), eukaryotic translation elongation factor 2 (EEEF2), reticulocalbin 3, EF-hand calcium binding domain (RCN2) and calponin 1 basic smooth muscle (CNN1) than that secreted by adherent cells from placenta or adipose tissue grown in a 2D culture.

35. The methods, population of cells or medium of claims 1, 2, 3, or 20, wherein said adherent cells express a lower level of expression of at least one protein selected from the group consisting of heterogeneous nuclear ribonucleoprotein H1 (Hnrph1), CD44 antigen isoform 2 precursor, 3 phosphoadenosine 5 phosphosulfate synthase 2 isoform a (Papss2) and ribosomal protein L7a (rpL7a) than that secreted by adherent cells from placenta or adipose tissue grown in a 2D culture.

36. The methods, population of cells or medium of claims 1, 2, 3, or 20, wherein said adherent cells or medium are characterized by a higher immunosuppressive activity than that of adherent cells of placenta or adipose tissue grown in a 2D culture.

37. The isolated population of cells of claim 36, wherein said immunosuppressive activity comprises reduction in T cell proliferation.

38. The method or cell population of any of claims 1, 2, 3, 4, 5, 6, 7, 12, or 13, wherein said cells comprise cells having a stromal stem cell phenotype.

39. The method of claim 38, wherein said stromal stem cell phenotype comprises T cell suppression activity.

40. The method of claim 38, wherein said stromal stem cell phenotype comprises hematopoietic stem cell support activity.

41. Use of the population of cells of claim 3, 4, 5, 6, or 7 for manufacture of a medicament identified for transplantation.

Fig. 1f

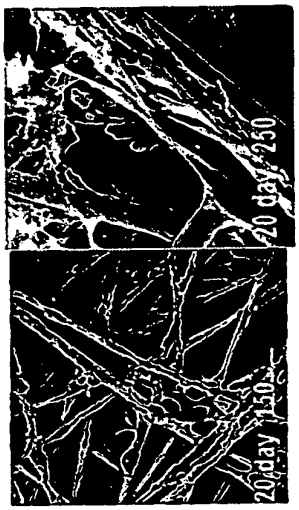


Fig. 1e

Fig. 1d

Fig. 1c

Fig. 1b

Fig. 1a

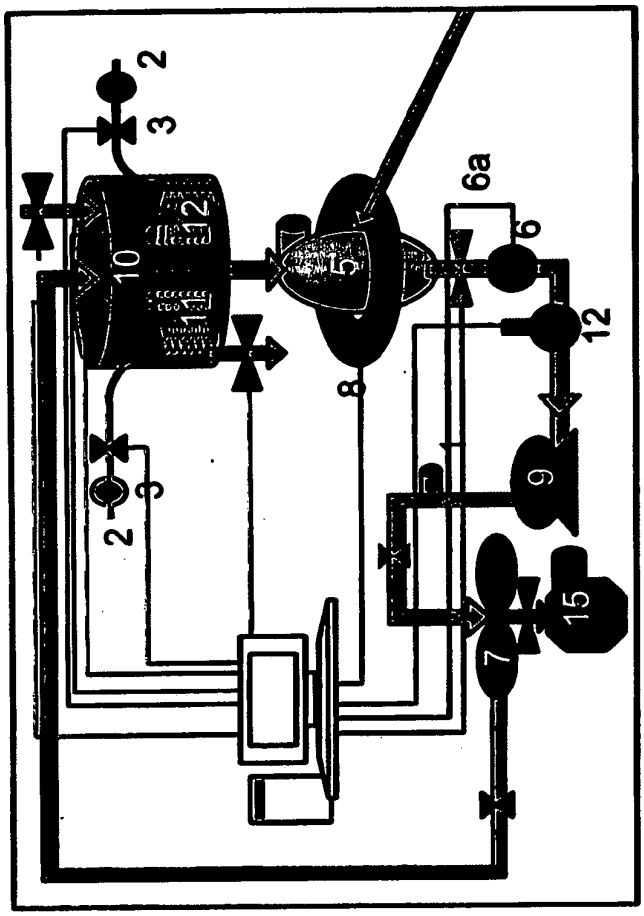
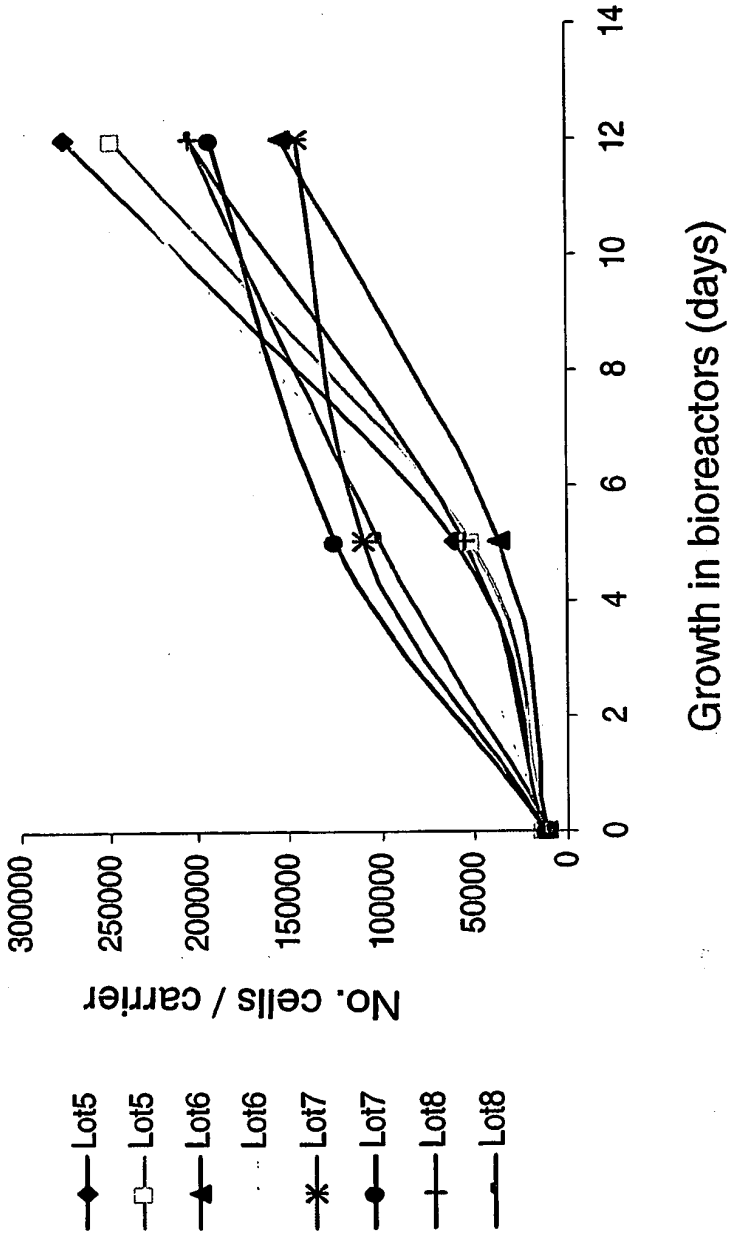


Fig. 1g

Fig. 2


No. cells / carrier
300000
250000
200000
150000
100000
50000
0
0
2
4
6
8
10
12
14
Growth in bioreactors (days)
Lot5
Lot5
Lot6
Lot6
Lot7
Lot7
Lot8
Lot8

Figs. 3a-b

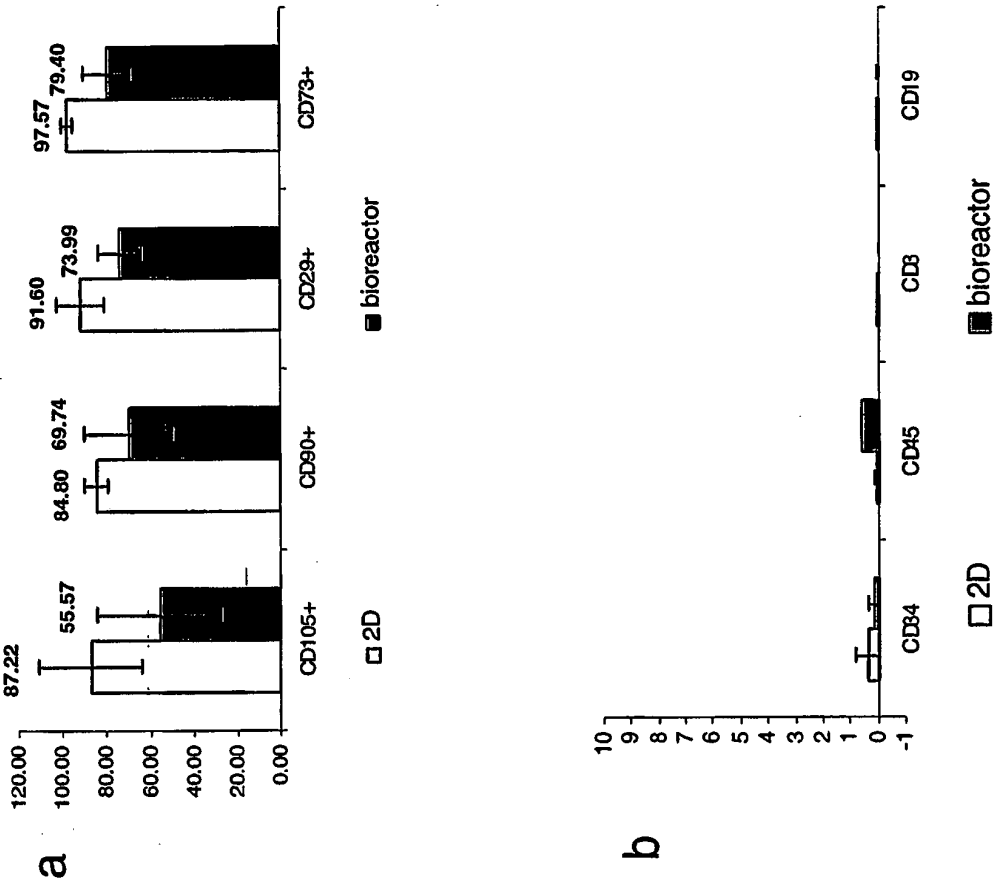

a
120.00
100.00
80.00
60.00
40.00
20.00
0.00
87.22
55.57
84.80
69.74
91.60
73.99
97.57
79.40
CD105+
CD90+
CD29+
CD73+
2D
bioreactor
b
10
9
8
7
6
5
4
3
2
1
0
-1
CD34
CD45
CD8
CD19
2D
bioreactor

Fig. 4a

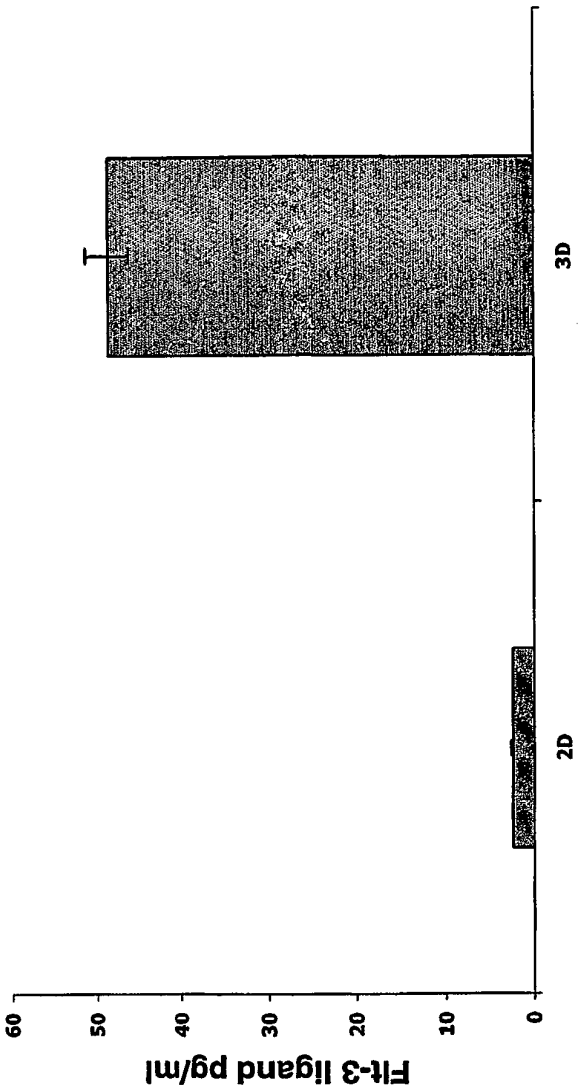


Fig. 4b

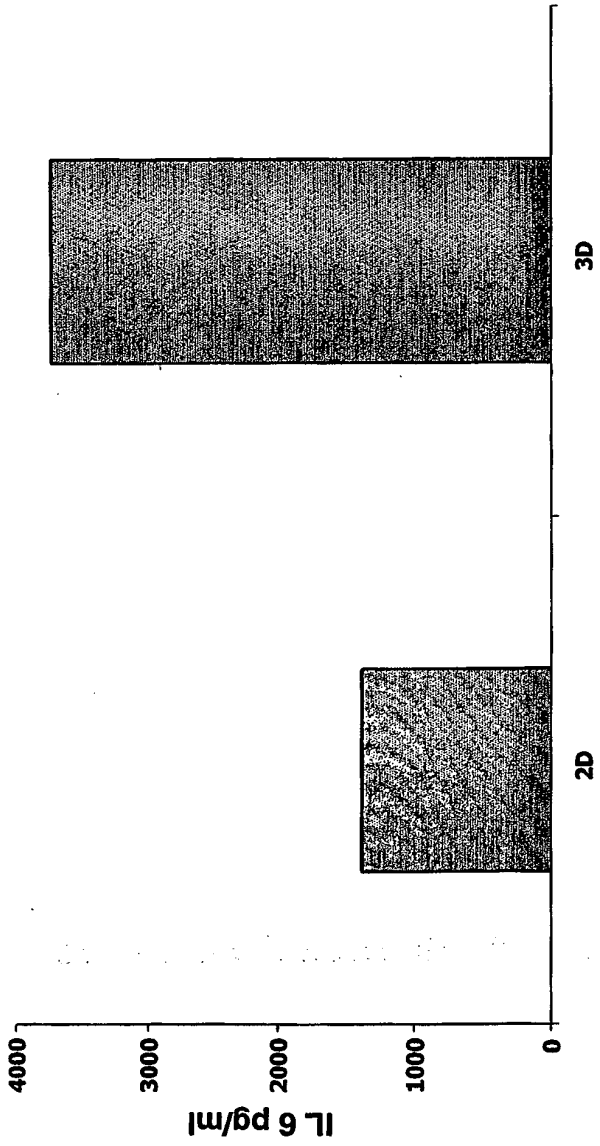
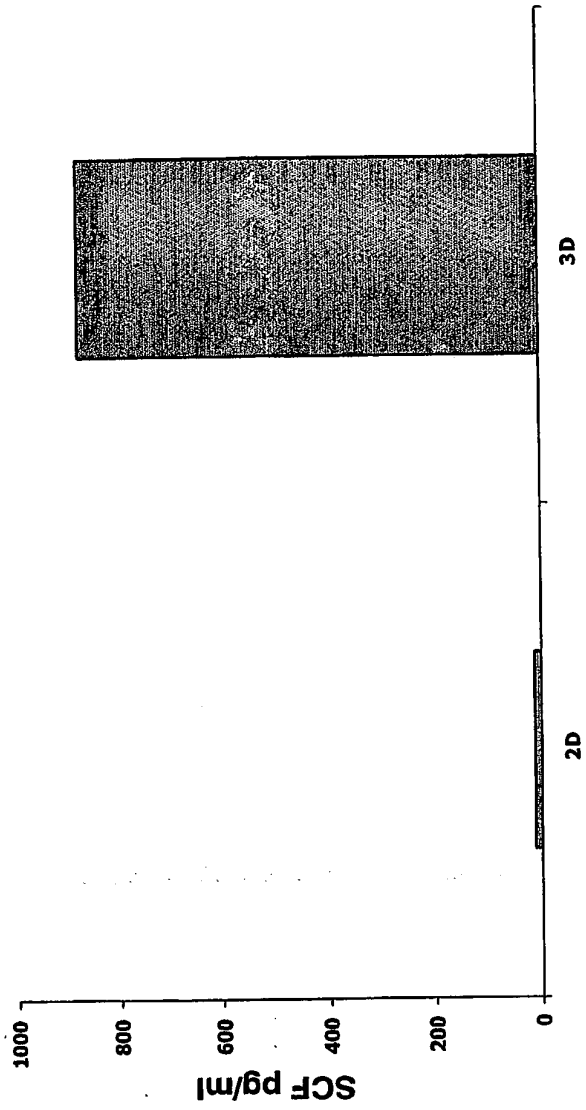


Fig. 4c



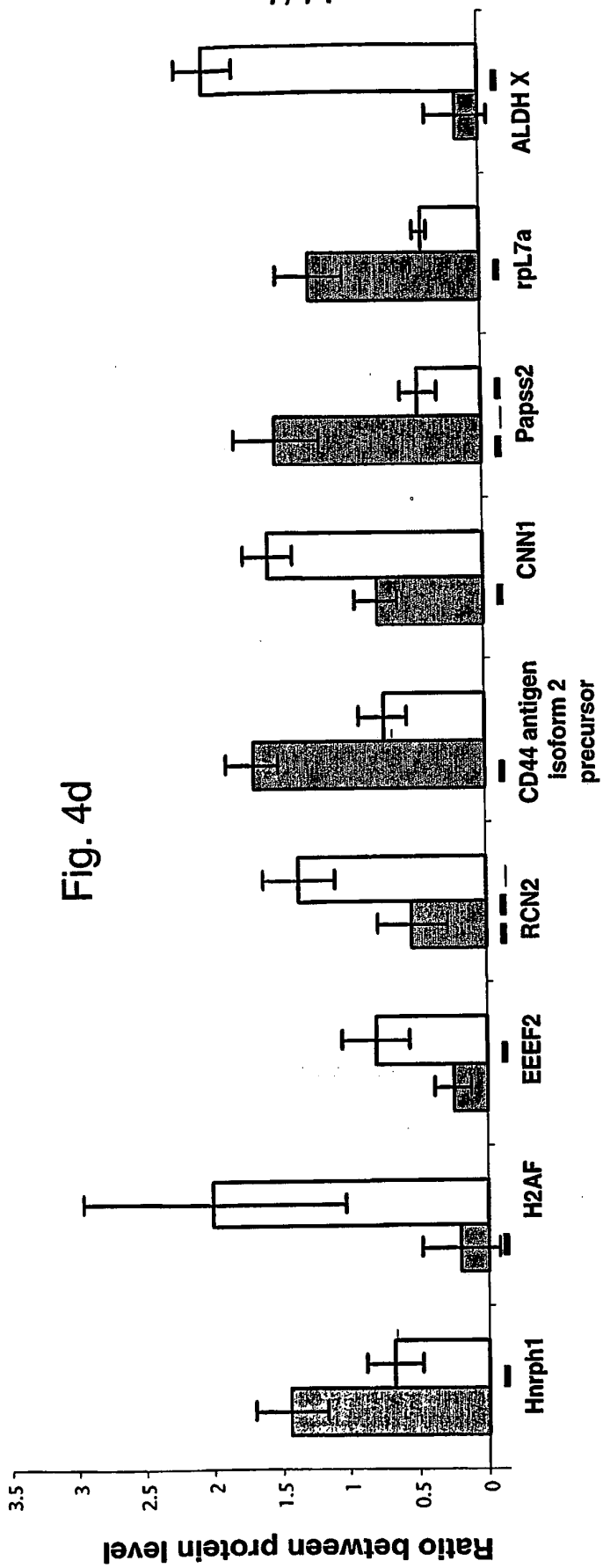
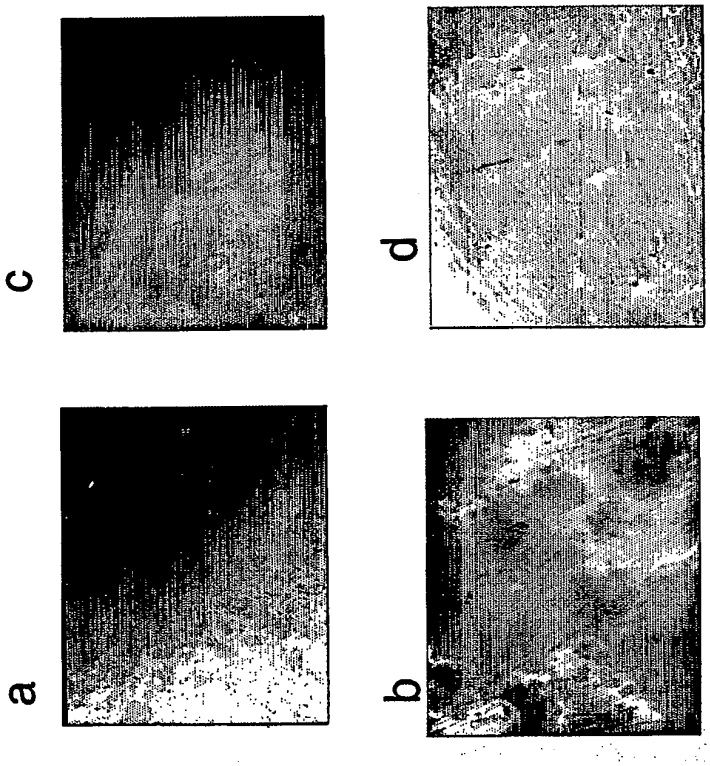

2D
3D
Ratio between protein level
3.5
3
2.5
2
1.5
1
0.5
0
Hnrph1
H2AF
EEEF2
RCN2
CD44 antigen isoform 2 precursor
CNN1
Papss2
rpL7a
ALDH X

Fig. 4d

Figs. 5a-d



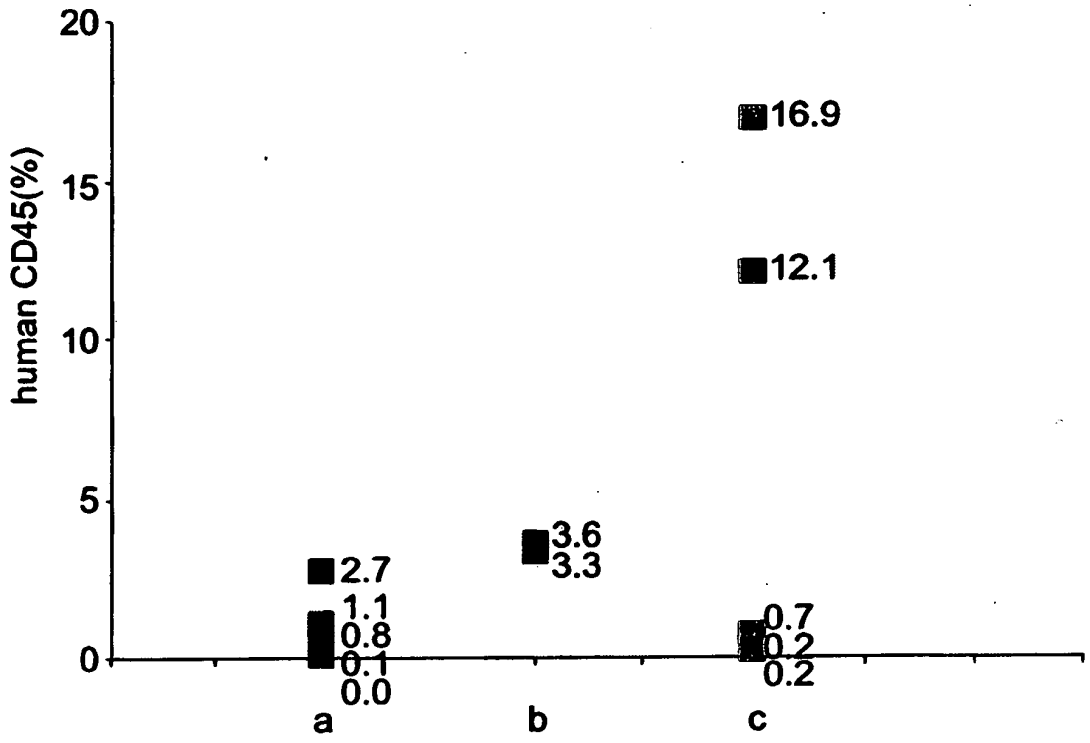


Fig. 6

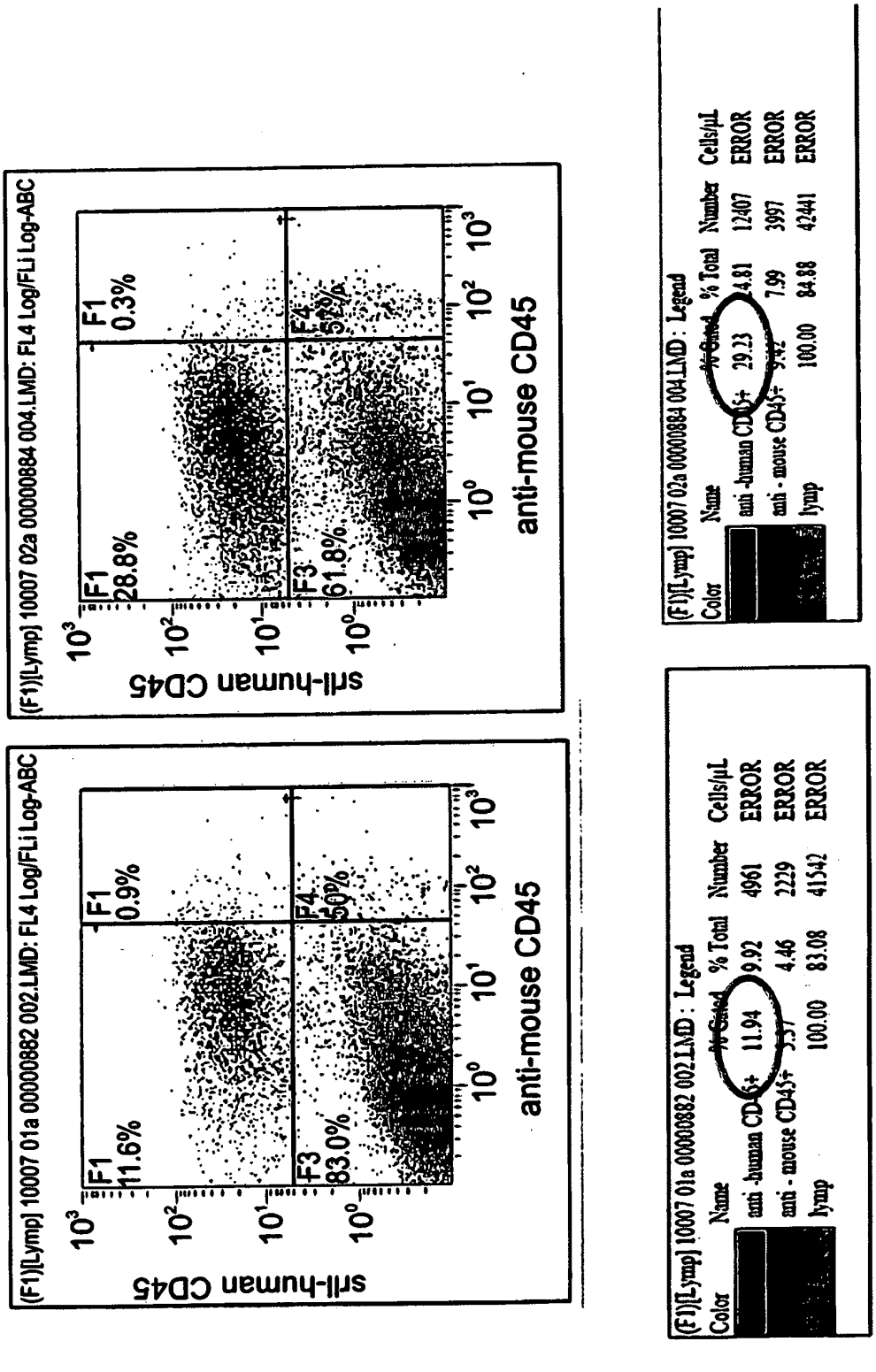
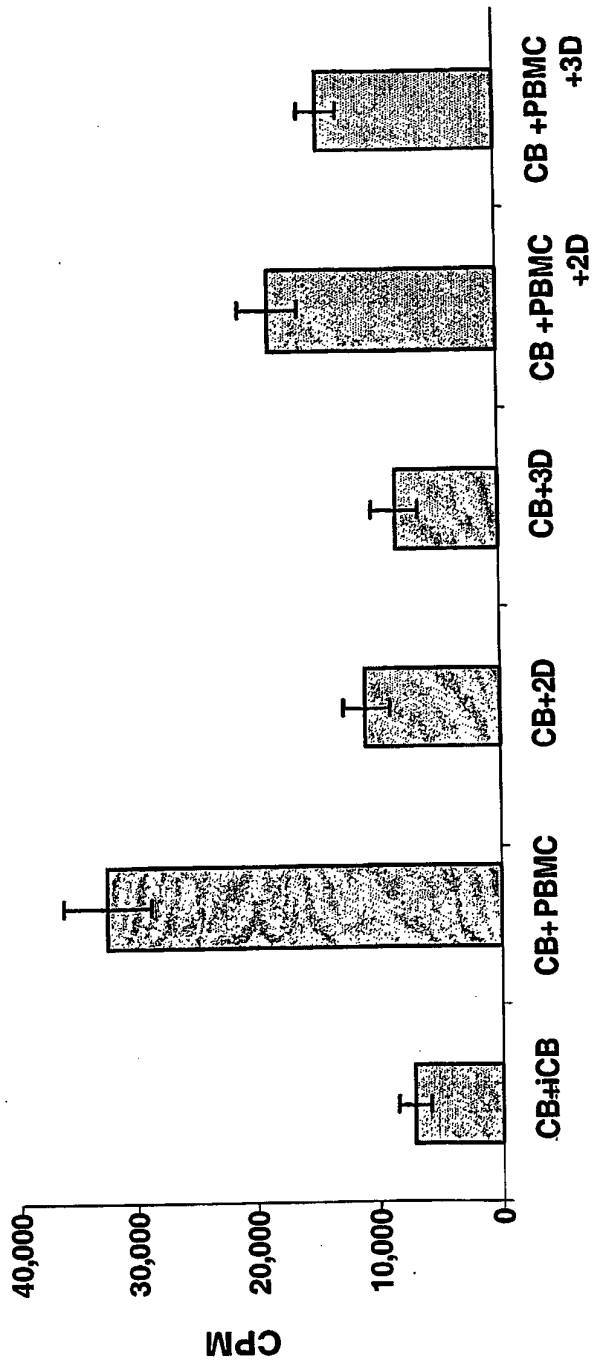


Fig. 7a

Fig. 7b

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

Fig. 8


CPM
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30,000
20,000
10,000
0
CB+iCB
CB+PBMC
CB+2D
CB+3D
CB +PBMC +2D
CB +PBMC +3D

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(71) Applicant *(for all designated States except US)*: **PLURISTEM LTD.** [IL/IL]; Matam Building # 20, 31905 Haifa (IL).

(71) Applicants and
(72) Inventors: **MERETZKI, Shai** [IL/IL]; 38 Raoul Wallenberg Street, 34990 Haifa (IL). **ABERMAN, Zami** [IL/IL]; 46 HaTapuz Street, 40600 Tel-Mond (IL). **BURGER, Ora** [IL/IL]; 5 Dulchin Street, 32882 Haifa (IL).

(74) Agents: **G.E. EHRLICH (1995) LTD.** et al.; 11 Menachem Begin Street, 52521 Ramat Gan (IL).

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(54) Title: METHODS FOR CELL EXPANSION AND USES OF CELLS AND CONDITIONED MEDIA PRODUCED THEREBY FOR THERAPY

(57) Abstract: A method of cell expansion is provided. The method comprising culturing adherent cells from placenta or adipose tissue under three-dimensional culturing conditions, which support cell expansion.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL07/00380.

A. CLASSIFICATION OF SUBJECT MATTER
IPC: C12N 5/00(2006.01)

USPC: 435/325,375,377
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S.: 435/325, 375, 377

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2003/0032179 A1 (HARIRI et al) 13 February 2003 (13.02.2003), paragraphs 16-24, 44-46, 63-114, and 135-149.	1, 3-19, 38-40 --- 2
X --- Y	US 2003/0235563 A1 (STROM et al) 25 December 2003 (25.12.2003), paragraphs 16, 20-25, 48, 49, 52, 58-65, and 99-109.	1, 3-19, 38-40 --- 2
X	US 2002/0076400 A1 (KATZ et al) 20 June 2002 (20.06.2002), paragraphs 6-14, 19, 20, and 26-51.	1-19, 38-40

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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

"E" earlier application or patent published on or after the international filing date

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

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

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

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

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 June 2008 (30.06.2008)	12 AUG 2008
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (571) 273-3201	Authorized officer Lora E. Barnhart Telephone No. 571-272-1600

Form PCT/ISA/210 (second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL07/00380

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.: 41
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 20-37
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

[19] 中华人民共和国国家知识产权局

[51] Int. Cl.
C12N 5/00 (2006.01)

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[71] 申请人 普拉里斯坦有限公司
地址 以色列海法
[72] 发明人 S·梅雷茨基 Z·阿伯曼
O·伯格
[74] 专利代理机构 中国专利代理(香港)有限公司
代理人 刘 冬 付 磊

权利要求书 4 页 说明书 40 页 附图 11 页

[54] 发明名称

细胞扩增方法和藉此产生的细胞和条件培养基用于治疗的用途

[57] 摘要

本发明提供细胞扩增方法。所述方法包括在支持细胞扩增的三维培养条件下培养来自胎盘和脂肪组织的粘附细胞。

1. 细胞扩增方法，所述方法包括在支持细胞扩增的三维培养条件下培养来自胎盘或脂肪组织的粘附细胞。

2. 产生条件培养基的方法，所述方法包括

(a)在允许细胞扩增的三维培养条件下培养来自胎盘或脂肪组织的粘附细胞；和

(b)收集所述扩增的粘附细胞的条件培养基，藉此产生条件培养基。

3. 权利要求 1 的方法产生的细胞群。

4. 分离的细胞群，其包含胎盘或脂肪组织粘附细胞，其中所述粘附细胞分泌比在 2D 培养中生长的胎盘或脂肪组织粘附细胞分泌的更高水平的选自 SCF、IL-6 和 Flt-3 的至少一种因子。

5. 分离的细胞群，其包含胎盘或脂肪组织粘附细胞，其中所述粘附细胞表达比在 2D 培养中生长的胎盘或脂肪组织粘附细胞表达的更高水平的选自 H2A 组蛋白家族(H2AF)、醛脱氢酶 X (ALDH X)、真核细胞翻译延伸因子 2 (EEEF2)、网钙结合蛋白 3、EF-手形钙结合结构域(RCN2)和钙调理蛋白 1 碱性平滑肌(CNN1)的至少一种蛋白质。

6. 分离的细胞群，其包含胎盘或脂肪组织粘附细胞，其中所述粘附细胞表达比在 2D 培养中生长的胎盘或脂肪组织粘附细胞表达的更低表达水平的选自核内不均一核糖核蛋白 H1 (Hnrph1)、CD44 抗原同种型 2 前体、3 磷酸腺苷 5 磷酰硫酸合成酶 2 同种型 a (Papss2)和核糖体蛋白 L7a (rpL7a)的至少一种蛋白质。

7. 分离的细胞群，其包含胎盘或脂肪组织粘附细胞，其中所述粘附细胞特征为比在 2D 培养中生长的胎盘或脂肪组织粘附细胞更高的免疫抑制活性。

8. 权利要求 7 的分离的细胞群，其中所述免疫抑制活性包含降低 T 细胞增殖。

9. 药物组合物，其包含作为活性成分的权利要求 1 产生的细胞群。

10. 药物组合物，其包含作为活性成分的权利要求 2 产生的条件培养基。

11. 药物组合物，其包含作为活性成分的权利要求 4、5、6 或 7 的分离的细胞群。

12. 治疗需要治疗的对象中可受益于基质细胞移植的病症的方法，所述方法包括给予所述对象治疗有效量的选自胎盘和脂肪组织的组织的粘附细胞，藉此治疗所述对象的所述可受益于干细胞移植的病症。

13. 治疗需要治疗的对象中可受益于基质细胞移植的病症的方法，所述方法包括给予所述对象治疗有效量的源自选自胎盘和脂肪组织的组织的粘附细胞的条件培养基，藉此治疗所述对象的所述可受益于干细胞移植的病症。

14. 降低有此需要的对象中免疫应答的方法，所述方法包括给予所述对象治疗有效量的权利要求 3、4、5、6 或 7 的分离的细胞群，以便降低所述对象的免疫应答。

15. 权利要求 14 的方法，其中用细胞疗法治疗所述对象。

16. 权利要求 12 或 13 的方法，其进一步包括给予干细胞。

17. 权利要求 16 的方法，其中所述干细胞包含造血干细胞。

18. 权利要求 16 的方法，其中所述干细胞与所述条件培养基或粘附细胞同时给予。

19. 权利要求 16 的方法，其中所述干细胞在给予所述条件培养基或粘附细胞之后给予。

20. 权利要求 12 或 13 的方法，其中所述粘附细胞得自三维培养。

21. 权利要求 12 或 13 的方法，其中所述粘附细胞得自二维培养。

22. 权利要求 12 或 13 的方法，其中所述病症选自干细胞缺乏症、心脏病、帕金森氏病、癌症、阿尔茨海默氏病、中风、烧伤、组织损

伤、失血、贫血症、自身免疫病、糖尿病、关节炎、多发性硬化症、移植物抗宿主病(GvHD)、神经退行性疾病、自身免疫性脑脊髓炎(EAE)、系统性红斑狼疮(SLE)、风湿性关节炎、系统性硬化症、Sjorgen综合征、多发性硬化症(MS)、重症肌无力(MG)、格-巴综合征(GBS)、桥本甲状腺炎(HT)、格雷夫斯病、胰岛素依赖性糖尿病(IDDM)和炎症性肠病。

23. 权利要求 1、2、3 或 20 的方法或细胞群，其中所述三维培养包含 3D 生物反应器。

24. 权利要求 23 的方法，其中所述生物反应器选自推流式生物反应器、连续搅拌槽生物反应器和固定床生物反应器。

25. 权利要求 1、2、3 或 20 的方法或细胞群，其中所述细胞的所述培养在连续流动培养基下实现。

26. 权利要求 1、2、3 或 20 的方法或细胞群，其中所述三维培养包含选自聚酯、聚链烯、聚氟氯乙烯、聚氯乙烯、聚苯乙烯、聚砜、醋酸纤维素、玻璃纤维、陶瓷颗粒、matrigel、细胞外基质组分、胶原、聚 L 乳酸和惰性金属纤维的粘附材料。

27. 权利要求 1、2、3 或 20 的方法或细胞群，其中所述培养至少进行 3 天。

28. 权利要求 21 的方法或细胞群，其中所述培养至少进行 3 天。

29. 权利要求 1、2、3 或 20 的方法或细胞群，其中进行所述培养直到所述粘附细胞达到至少 60 %汇合。

30. 权利要求 12 或 13 的方法，其中所述病症可受益于对造血干细胞移入的促进。

31. 权利要求 1、2、3、4、5、6、7 或 20 的方法、细胞群或培养基，其中所述粘附细胞包含选自 CD73、CD90、CD29 和 CD105 的阳性标记表达系列。

32. 权利要求 1、2、3、4、5、6、7 或 20 的方法、细胞群或培养基，其中所述粘附细胞包含选自 CD45、CD80、HLA-DR、Cd11b、

CD14、CD19、CD34 和 CD79 的阴性标记表达系列。

33. 权利要求 1、2、3 或 20 的方法、细胞群或培养基，其中所述粘附细胞分泌比在 2D 培养中生长的来自胎盘或脂肪组织的粘附细胞分泌的更高水平的选自 SCF、Flt-3 和 IL-6 的至少一种因子。

34. 权利要求 1、2、3 或 20 的方法、细胞群或培养基，其中所述粘附细胞表达比在 2D 培养中生长的来自胎盘或脂肪组织的粘附细胞分泌的更高水平的选自 H2A 组蛋白家族(H2AF)、醛脱氢酶 X (ALDH X)、真核细胞翻译延伸因子 2 (EEEF2)、网钙结合蛋白 3、EF-手形钙结合结构域(RCN2)和钙调理蛋白 1 碱性平滑肌(CNN1)的至少一种蛋白质。

35. 权利要求 1、2、3 或 20 的方法、细胞群或培养基，其中所述粘附细胞表达比在 2D 培养中生长的来自胎盘或脂肪组织的粘附细胞分泌的更低表达水平的选自核内不均一核糖核蛋白 H1 (Hnrph1)、CD44 抗原同种型 2 前体、3 磷酸腺苷 5 磷酰硫酸合成酶 2 同种型 a (Papss2)和核糖体蛋白 L7a (rpL7a)的至少一种蛋白质。

36. 权利要求 1、2、3 或 20 的方法、细胞群或培养基，其中所述粘附细胞或培养基特征为比在 2D 培养中生长的胎盘或脂肪组织粘附细胞更高的免疫抑制活性。

37. 权利要求 36 的分离的细胞群，其中所述免疫抑制活性包含降低 T 细胞增殖。

38. 权利要求 1、2、3、4、5、6、7、12 或 13 的方法或细胞群，其中所述细胞包含具基质干细胞表型的细胞。

39. 权利要求 38 的方法，其中所述基质干细胞表型包含 T 细胞抑制活性。

40. 权利要求 38 的方法，其中所述基质干细胞表型包含造血干细胞支持活性。

41. 权利要求 3、4、5、6 或 7 中的细胞群在制备用于鉴定移植的药物中的用途。

细胞扩增方法和藉此产生的细胞和条件培养基用于治疗的用途

发明领域和发明背景

本发明涉及细胞扩增方法、藉此产生的细胞群及其用途。具体地本发明涉及扩增来自胎盘或脂肪组织(贯穿于整个 PCT 中)的粘附细胞(adherent cell)的方法及其例如用于造血干细胞移植的治疗用途。

在日益发展的医学界，为了细胞移入和组织工程目的越来越需要大量成人干细胞。另外，成人干细胞疗法正持续不断地开发用于治疗和治愈各种病症，例如造血功能障碍、心脏病、帕金森氏病、阿尔茨海默氏病、中风、烧伤、肌肉萎缩症、自身免疫病、糖尿病和关节炎。

造血干细胞(HSC)是祖细胞，其产生髓系和淋巴系的所有血细胞类型。移植的 HSC 的移入和启动造血作用取决于 HSC 在受者骨髓中的归巢和增生的能力。

普遍公认干细胞在体内与骨髓中离散的龛(niche)有密切的联系，所述龛提供了经由细胞间接触或短距离作用共同介导干细胞分化和自我更新的分子信号。这些龛是“造血诱导性微环境”(HIM)的部分，由骨髓细胞即巨噬细胞、成纤维细胞、脂肪细胞和内皮细胞组成。骨髓细胞通过提供促进细胞间接触的细胞外基质(ECM)蛋白和基底膜组分来保持 HIM 功能完整性。它们还提供用于控制造血细胞分化和增殖所需的各种可溶性或常驻性细胞因子。

需要 HSC 和基质之间的相互作用以保护 HSC 生存力并防止其分化。在 HSC 移植后，移植的 HSC 在其增殖和分化之前必须归巢到骨髓(BM)微环境并驻留在合适龛中。在归巢过程中，移植的 HSC 离开血流，并通过随趋化因子梯度穿过 BM 内皮细胞屏障到达专用龛来转移。然后供者 HSC 必须归巢到造血龛，在那里它们遇到对其分裂更有利的微环境，在那里 HSC 与间充质细胞、ECM 和分泌的生长因子之

间可建立连续统一的物理和化学接触。所有这些过程涉及一系列复杂的分子，例如细胞因子、趋化因子、激素、类固醇、细胞外基质蛋白、生长因子、细胞间相互作用蛋白、黏附蛋白和基质蛋白。

移入到 BM 专用龛的细胞总数构成 HSC 移植成功的基础。为了完成移入，移植到血液循环的供者 HSC 应该归巢到其产生功能性血细胞生成集落(hematopoiesis foci)的受者骨髓中。这些灶的数目可从输注的 HSC 总数乘以其移入效率的积来推算。

HSC 移植涉及的主要问题之一是这些细胞在接受者系统中存活率低。充分证明的是，静脉内移植的 HSC 被从循环中清除并在其灌输后数分钟内在 BM 中显现。HSC 移植后 3-5 小时，在受者的外周血中检测不到供者细胞[Askenasy 等 2002，移植的造血细胞在体内丛生于受者骨髓中(Transplanted hematopoietic cells seed in clusters in recipient bone marrow in vivo). Stem Cells. 20:301-10]。灌输后不久大量的移植细胞被破坏。因此，受者骨髓建群有效性低，移植后 2-3 天在受者 BM 中检测到的所输注细胞仅为 1-5% [Kerre 等 2001，CD34+38+和 CD34+38-两种细胞特异性归巢到 NOD/LtSZ scid/scid 小鼠骨髓但显示不同的扩增动力学(Both CD34+38+ and CD34+38- cells home specifically to the bone marrow of NOD/LtSZ scid/scid mice but show different kinetics in expansion). J Immunol. 167:3692-8; Jetmore 等 2002，移植到条件化 NOD/SCID 受者中的静止和循环的人 CD34(+)细胞的归巢效率、细胞周期动力学和存活率(Homing efficiency, cell cycle kinetics, and survival of quiescent and cycling human CD34(+) cells transplanted into conditioned NOD/SCID recipients). Blood. 99:1585-93]。

间充质基质细胞(Mesenchymal Stromal Cell) (MSC)为异质细胞群，能够分化为不同类型的成熟间充质细胞。这些细胞受到各种生物活性因子影响而分化为网状内皮细胞、成纤维细胞、脂肪细胞和骨祖细胞(osteogenic precursor cell)。

MSC 支持 HSC 移入的用途已为本领域所知。若干出版物已经证

明当与间充质干细胞共同移植时，HSC 的移入效率更高 [Gurevitch 等 1999，在供体基质微环境中同种异体或异种骨髓移植(Transplantation of allogeneic or xenogeneic bone marrow within the donor stromal microenvironment). Transplantation. 68:1362-8; Fan 等 2001，通过经门静脉注射骨髓细胞成功实施同种异体骨髓移植(BMT): 基质细胞作为促进 BMT 的细胞(Successful allogeneic bone marrow transplantation (BMT) by injection of bone marrow cells via portal vein: stromal cells asBMT-facilitating cells). Stem Cells. 19:144-50]。还证明了在人羊移入模型中共同移植人间充质干细胞导致促进人 HSC 嵌合 BM 在动物中的长期移入[Almeida-Porada 等 2000，将人基质细胞祖先共同移植到免疫前的胎羊中导致在循环中早期出现人供者细胞并在移植后的稍后时间点提高骨髓中的细胞水平(Co-transplantation of human stromal cell progenitors into preimmune fetal sheep results in early appearance of human donor cells in the circulation and boosts cell levels in bone marrow at later time points after transplantation). Blood. 95:3620-7]。发现同时注射 HSC 和间充质干细胞加速血细胞生成 [Zhang 等 2004，Stem Cells. 22:1256-62]。最近，这些发现扩展到更近缘的动物模型—猕猴。当共同移植单倍体同一性(haploidentical)的 HSC 和间充质干细胞时，证明是促进了 HSC 移入[Liu 等 2005，Zhonghua Xue Ye Xue Za Zhi. 26:385-8]。最近还报道了使用间充质干细胞来促进 HSC 移入人类对象[Koc ON, J Clin Oncol. 2000;18:307-316: Lazarus HM, Biol Blood Marrow Transplant. 2005 May; 11(5):389-98]。

显然，MSC 对造血移入的贡献在于：产生帮助介导并平衡所移植 HSC 的归巢、自我更新和定向潜能的 HSC 支持性细胞因子；复原受损的 HSC 归巢和增殖所需造血微环境；以及抑制源自供者的 T 细胞(可引起移植物抗宿主病(GvHD))，[Charbord P.和 Moore, K., *Ann. N. Y. Acad. Sci.* 1044: 159-167(2005)；美国专利第 6,010,696 号；第 6555374 号]。例如，在 Maitra 的研究中[Maitra B 等，Bone Marrow Transplant. 33(6):597-604.(2004)]发现在 NOD-SCID 小鼠模型中，人间充质干细胞

支持非亲缘供者造血干细胞和受抑制的 T 细胞激活，表明非亲缘源自人骨髓的 MSC 可改进同种异体移植结果。

使用 MSC 的一个主要障碍是难以分离大量的这些正常存在的细胞群，其从技术上来说是困难的且耗费大，部分是由于细胞数量有限。MSC 最明显的来源是骨髓，但涉及获得骨髓吸出物和活检风险的显著不便之处成为这些方法的缺点。人胚胎和胎儿形成独立生命这一普遍持有观点使得人胚胎作为干细胞来源有了疑问，在已经存在的逻辑困难之上又增加了宗教和伦理方面的问题。

近来已在尝试从备选来源中发现可收获的干细胞。这样的备选来源有例如：脂肪组织、毛囊、睾丸、人嗅粘膜、胚胎卵黄囊、胎盘、青少年皮肤和血液(例如脐带血甚至月经血)。然而，从备选来源收获足够量的干细胞用于治疗和研究目的仍受限制且通常是费事的，其涉及例如从供者对象或患者收获细胞或组织、体外培养和/或繁殖细胞、解剖(dissection)等等。

胎盘被认为是干细胞最有可能的来源之一，不涉及任何不便之处或伦理约束。发现源自胎盘的 MSC 具有与源自 BM 的 MSC 相似的特性。它们能粘附塑料，表达 CD105、CD73 和 CD90 膜标记，且缺乏 CD45、CD34、CD14、CD19 和 HLA-DR 表面分子的表达。然而，不象源自 BM 的 MSC，经干扰素-γ 处理的源自胎盘的 MSC (placenta derived-MSC, PD-MSC)极低限度地上调 HLA-DR。此外，PD-MSC 细胞在干扰素-γ 存在下显示出提高的免疫抑制特性。(Chang CJ, Yen ML, Chen YC, Chien CC, Huang HI, Bai CH, Yen BL. 在干扰素-γ 存在下源自胎盘多潜能细胞显示出提高的免疫抑制特性 (Placenta-derived Multipotent Cells exhibit immunosuppressive properties that are enhanced in the presence of interferon- gamma). Stem Cells. 2006 Nov;24(11):2466-77.)。

除 MSC 标记之外，PD-MSC 还显示出独特的 ESC 表面标记 SSEA-4、TRA-1-61 和 TRA-1-80，提示这些可能是极为原始的细胞。(Yen BL, Huang HI, Chien CC, Jui HY, Ko BS, Yao M, Shun CT, Yen

ML, Lee MC, Chen YC. 从人足月胎盘分离多潜能细胞(Isolation of multipotent cells from human term placenta). Stem Cells. 2005; 23(l): 3-9)。此外，PD-MSC(胎儿来源)而非源自 BM 的 MSC 对于细胞内人白细胞抗原-G (HLA)为阳性(Chang CJ, Yen ML, Chen YC, Chien CC, Huang HI, Bai CH, Yen BL，在干扰素-γ存在下源自胎盘多潜能细胞显示出提高的免疫抑制特性(Placenta-derived Multipotent Cells exhibit immunosuppressive properties that are enhanced in the presence of interferon- gamma). Stem Cells. 2006 Nov;24(l l):2466-77.)。

研究表明 PD-MSC 的扩增潜能显著高于源自成人 BM 的 MSC (Yen BL, Huang HI, Chien CC, Jui HY, Ko BS, Yao M, Shun CT, Yen ML, Lee MC, Chen YC，从人足月胎盘分离多潜能细胞(Isolation of Multipotent cells from Human Term Placenta). Stem Cells. 2005;23(1):3-9; M.J.S. de Groot-Swings, Frans H.J. Claas, Willem E. Fibbe 和 Humphrey H.H. Pieternella S. in 't Anker, Sicco A. Scherjon, Carin Kleijburg-van der Keur, Godelieve. 从人中胎盘分离胎儿或母体来源的间充质干细胞(Placenta Isolation of Mesenchymal Stem Cells of Fetal or Maternal Origin from Human). *Stem cells*,2004;22;1338-1345)。另外，源自胎盘的粘附细胞可分化为成骨细胞、脂肪细胞和成软骨细胞。发现源自胎盘的 MSC 如源自 BM 的 MSC 一样阻抑脐带血(UCB)淋巴细胞增殖，这表明联合移植 HSC 和源自胎盘(PD)-MSC 可降低受者潜在的移植物抗宿主病(GvHD) [Li CD 等，Cell Res. Jul;15(7):539-47 (2005)]，并且可增强造血支持[Zhang Yi 等，Chinese Medical Journal 117(6): 882-887(2004)]。胎盘作为羊膜上皮细胞来源的应用教导于例如 WO 00/73421 中，但得到这些细胞仍很麻烦，并且 MSC 产率极低。

解决 MSC 量有限问题的另一方法是用不同的培养条件离体扩增这些细胞[例如美国专利第 6,326,198 号; 第 6030836 号; 第 6555374 号; 第 6,335,195 号; 第 6,338,942 号]。然而，这样的方法的缺点仍在于耗时、需要特定选择和分离程序，使得这些方法花费大、难实施。

在若干研究中发现三维(3D)细胞培养在产量上更为有效[Ma T

等，生物工程进展(Biotechnology Progress). Biotechnol Prog 15:715-24(1999); Yubing Xie, Tissue Engineering 7(5): 585-598(2001)]。模拟MSC天然环境的3D培养方法的应用基于将这些细胞接种到以下生物反应器中：含有 Polyactive 泡沫的灌注式生物反应器[Wendt, D. 等，Biotechnol Bioeng 84: 205-214,(2003)]、管状聚L乳酸(PLLA)多孔支架的径向流灌注式生物反应器[Kitagawa 等，Biotechnology and Bioengineering 93(5): 947-954(2006)]、和用于培养和扩增造血干细胞的推流式生物反应器(美国专利第 6,911,201 号)。

在美国专利第 6,022,743 号中提出粘附基质细胞的三维框架，在 Hosseinkhani, H 等，[Tissue Engineering 11(9-10): 1476-1488(2005)]中提出海绵胶原作为 3D 基质。然而，这些研究中无一曾提及在这些条件下生长的 MSC 用于在 HSC 移植后支持体内移入 HSC 的用途。还需要耗时来为特定细胞类型优化各种条件(例如灌注条件)或各种分离技术。

在美国专利第 7045148 号和美国专利申请第 20020123141 号、第 20030032179 号和第 2005011871 号中提出灌注的产后胎盘作为 3D 反应器用于培养 MSC 的用途。然而，该方法受限于要待到胎盘分离后 24 小时并涉及灌注，因此，细胞的群集生长(mass growth)且其在延长的时间周期内维持是不可能的。

因此，普遍公认需要且将极为有利的是具备：不存在上述限制的新颖的细胞扩增方法和藉此产生的细胞及条件培养基用于治疗的用途。

发明简述

本发明一方面提供细胞扩增方法，所述方法包括在支持细胞扩增的三维培养条件下培养来自胎盘或脂肪组织的粘附细胞。

本发明另一方面提供产生条件培养基的方法，所述方法包括：在允许细胞扩增的三维培养条件下培养来自胎盘或脂肪组织的粘附细

胞；和收集扩增的粘附细胞的条件培养基，藉此产生条件培养基。

本发明又一方面提供根据上述方法产生的细胞群。

本发明再一方面提供分离的细胞群，其包含胎盘或脂肪组织粘附细胞，其中所述粘附细胞分泌比在 2D 培养中生长的胎盘或脂肪组织粘附细胞分泌的更高水平的选自 SCF、IL-6 和 Flt-3 的至少一种因子。

本发明另外方面提供分离的细胞群，其包含胎盘或脂肪组织粘附细胞，其中所述粘附细胞表达比在 2D 培养中生长的胎盘或脂肪组织粘附细胞表达的更高水平的选自 H2A 组蛋白家族(H2AF)、醛脱氢酶 X (ALDH X)、真核细胞翻译延伸因子 2 (EEEF2)、网钙结合蛋白 3、EF-手形钙结合结构域(RCN2)和钙调理蛋白 1 碱性平滑肌(CNN1)的至少一种蛋白质。

本发明又一方面提供分离的细胞群，其包含胎盘或脂肪组织粘附细胞，其中所述粘附细胞表达比在 2D 培养中生长的胎盘或脂肪组织粘附细胞表达的更低表达水平的选自核内不均一核糖核蛋白 H1 (Hnrph1)、CD44 抗原同种型 2 前体、3 磷酸腺苷 5 磷酰硫酸合成酶 2 同种型 a (Papss2)和核糖体蛋白 L7a (rpL7a)的至少一种蛋白质。

本发明再一方面提供分离的细胞群，其包含胎盘或脂肪组织粘附细胞，其中所述粘附细胞特征为比在 2D 培养中生长的胎盘或脂肪组织粘附细胞的免疫抑制活性更高。

根据下述本发明优选实施方案的另外特征，免疫抑制活性包含降低 T 细胞增殖。

本发明另一方面提供药物组合物，其包含作为活性成分的按照上述方法产生的细胞群。

本发明另一方面提供药物组合物，其包含作为活性成分的按照上述方法产生的条件培养基。

本发明又一方面提供药物组合物，其包含作为活性成分的上述分离的细胞群。

本发明再一方面提供治疗有其需要的对象中可受益于基质细胞

移植的病症的方法，所述方法包括：给予该对象治疗有效量的选自胎盘和脂肪组织的组织的粘附细胞，藉此治疗该对象中可受益于干细胞移植的病症。

本发明再一方面提供治疗有其需要的对象中可受益于基质细胞移植的病症的方法，所述方法包括：给予该对象治疗有效量的源自选自胎盘和脂肪组织的组织的粘附细胞的条件培养基，藉此治疗对象中可受益于干细胞移植的病症。

本发明再一方面提供降低有其需要的对象中的免疫应答的方法，所述方法包括给予该对象治疗有效量的权利要求 3、4、5、6 或 7 的分离的细胞群，以此降低该对象的免疫应答。

根据所述优选实施方案的再一特征，用细胞疗法治疗该对象。

根据所述优选实施方案的再一特征，该方法进一步包括给予干细胞。

根据所述优选实施方案的再一特征，该干细胞包含造血干细胞。

根据所述优选实施方案的再一特征，将所述细胞与条件培养基或粘附细胞同时给予。

根据所述优选实施方案的再一特征，在给予条件培养基或粘附细胞后给予所述细胞。

根据所述优选实施方案的再一特征，该粘附细胞从三维培养获得。

根据所述优选实施方案的再一特征，该粘附细胞从二维培养获得。

根据所述优选实施方案的再一特征，所述病症选自：干细胞缺乏症、心脏病、帕金森氏病、癌症、阿尔茨海默氏病、中风、烧伤、组织缺损(loss of tissue)、失血、贫血症、自身免疫病、糖尿病、关节炎、多发性硬化症、移植物抗宿主病(GvHD)、神经退行性疾病、自身免疫性脑脊髓炎(EAE)、系统性红斑狼疮(SLE)、类风湿性关节炎、系统性硬化症、Sjorgen 综合征、多发性硬化症(MS)、重症肌无力(MG)、格-

巴综合征(GBS)、桥本甲状腺炎(HT)、格雷夫斯病、胰岛素依赖性糖尿病(IDDM)和炎症性肠病。

根据所述优选实施方案的再一特征，所述三维培养包含 3D 生物反应器。

根据所述优选实施方案的再一特征，所述生物反应器选自推流式生物反应器、连续搅拌罐生物反应器和固定床生物反应器。

根据所述优选实施方案的再一特征，细胞培养在连续流动培养基中进行。

根据所述优选实施方案的再一特征，三维培养包含粘附材料选自：聚酯、聚链烯、聚氟氯乙烯、聚氯乙烯、聚苯乙烯、聚砜、醋酸纤维素、玻璃纤维、陶瓷颗粒、matrigel (基质胶)、细胞外基质组分、胶原、聚 L 乳酸和惰性金属纤维。

根据所述优选实施方案的再一特征，所述培养至少进行 3 天。

根据所述优选实施方案的再一特征，所述培养至少进行 3 天。

根据所述优选实施方案的再一特征，直到粘附细胞达到至少 60% 汇合(confluence)才完成所述培养。

根据所述优选实施方案的再一特征，所述病症可受益于对造血干细胞移入的促进。

根据所述优选实施方案的再一特征，所述粘附细胞包含选自 CD73、CD90、CD29 和 CD105 的阳性标记表达系列(marker expression array)。

根据所述优选实施方案的再一特征，所述粘附细胞包含选自 CD45、CD80、HLA-DR、CD11b、CD14、CD19、CD34 和 CD79 的阴性标记表达系列。

根据所述优选实施方案的再一特征，所述粘附细胞分泌比在 2D 培养中生长的来自胎盘或脂肪组织的粘附细胞分泌的更高水平的选自 SCF、Flt-3 和 IL-6 的至少一种因子。

根据所述优选实施方案的再一特征，所述粘附细胞表达比在 2D

培养中生长的来自胎盘或脂肪组织的粘附细胞分泌的更高水平的选自 H2A 组蛋白家族(H2AF)、醛脱氢酶 X (ALDH X)、真核细胞翻译延伸因子 2 (EEEF2)、网钙结合蛋白 3、EF-手形钙结合结构域(RCN2)和钙调理蛋白 1 碱性平滑肌(CNN1)的至少一种蛋白质。

根据所述优选实施方案的再一特征，所述粘附细胞表达比在 2D 培养中生长的来自胎盘或脂肪组织的粘附细胞分泌的更低表达水平的选自核内不均一核糖核蛋白 H1 (Hnrph1)、CD44 抗原同种型 2 前体、3 磷酸腺苷 5 磷酰硫酸合成酶 2 同种型 a (Papss2)和核糖体蛋白 L7a (rpL7a)的至少一种蛋白质。

根据所述优选实施方案的再一特征，所述粘附细胞或培养基特征为比在 2D 培养中生长的胎盘或脂肪组织粘附细胞免疫抑制活性更高。

根据所述优选实施方案的再一特征，所述免疫抑制活性包含降低 T 细胞增殖。

根据所述优选实施方案的再一特征，所述细胞包含具有基质干细胞表型的细胞

根据所述优选实施方案的再一特征，所述基质干细胞表型包含 T 细胞抑制活性。

根据所述优选实施方案的再一特征，所述基质干细胞表型包含造血干细胞支持活性。

根据所述优选实施方案的再一特征，上述细胞群的用途为制备用于鉴定移植的药物。

本发明通过提供新颖的细胞扩增方法和藉此产生的细胞和条件培养基用于治疗的用途，成功解决目前已知结构(configuration)中的缺点。

除非另外定义，否则本文所用的所有科技术语具有与本发明所属领域一般技术人员通常理解相同的含义。在本发明实践或试验中，尽管可使用与本文所述相似或相当的方法和材料，但适宜的方法和材料

如下文所述。在有冲突的情况下，包括定义在内的本专利说明书将起主导作用。另外，材料、方法和实施例仅为说明性的，并非意欲限制。

附图简述

本发明于此仅通过与附图有关的实例来阐述。强调的是，于此与具体附图有关的详细内容通过实施例出示且仅旨在阐述性地讨论本发明的优选实施方案，并且是为了提供什么被认为是本发明原理和构思方面的最有用和最易理解的说明而提出。在这一点上，不试图比基本理解本发明所需的更详细地显示本发明结构性细节，对附图所作的说明使得本领域技术人员明白本发明的几种形式在实践中是怎样具体化的。

在附图中：

图 1a-g 描述在含有 3-D 载体的生物反应器系统中创造的类骨微环境。图 1a-b 是描述天然骨(图 1a)与在接种粘附基质细胞(3D-ASC)后 7 天 PluriX™ 3D 载体结构的仿造骨微环境(图 1b)的比较电子显微图。图 1c-f 是描述用从骨髓产生的 3D-ASC 接种的 PluriX™3D 基质在接种后 20 天(图 1c-d，分别放大 X 150 和 250)和 40 天(图 1e-f，分别放大 X 350 和 500)的电子显微图。图 1g 是具有由编号定义的单独部件的 Plurix 3D 推流式生物反应器：培养基库(1)、混合气体供给(2)、过滤器(3)、注射点(4)、在其中放置 3D 载体的柱(5)、流量监控器(6)、流量阀(6a)、分离容器(7)、细胞生长分析仪(8)、蠕动泵(9)、取样点(10)、溶 O_2 测量电极(11)、pH 测量电极(12)、控制系统(13)、新鲜生长培养基(14)、用过的生长培养基(15)。

图 2 是描述在生物反应器系统内 3D 生长条件下生长的来源于胎盘的粘附基质细胞不同生产批次(lot)图(3D-ASC; 批次 5-8)。将 ASC (2 X 10^6)以 10000 – 15000 个细胞/载体的密度接种到生物反应器。培养 12 天后，3D-ASC 的密度达到 150,000-250,000 个细胞/载体或含有 150 个载体的生物反应器中 22.5-37.5 X 10^6 个。。

图 3a-b 是描述比较在源自胎盘的 3D-ASC 中表达的膜标记(暗紫色)与在常规 2D 培养条件下培养的胎盘细胞中的膜标记(淡紫色)的表达水平差异的条形图。粘附细胞在培养瓶(2D)中生长 4-6 周，或在生物反应器系统中于聚苯乙烯载体(3D)上生长 2-3 周。使细胞从培养瓶或载体经收获后，孵育并与识别 MSC (图 3a)或造血细胞(图 3b)膜标记特征的一组单克隆抗体(MAb)结合。注意到在 2D 培养细胞中的 MSC 膜标记表达(如对于 CD90、CD 105、CD73 和 CD29 膜标记所示)比在 3D 培养的粘附细胞中表达的 MSC 膜标记明显更高，尤其是 CD105，在 3D 培养细胞中显示 56%表达，相比较在 2D 培养细胞中为 87%(图 3a)。2D 和 3D 二者培养物中的 ASC 都不表达任何造血膜标记(图 3b)。

图 4a-d 是描述比较在 2D 和 3D 条件或在 2D 和 3D 条件培养基下培养的从胎盘产生的 ASC 的蛋白质水平的条形图。图 4a-c 描述由 ELISA 分析以 pg/ml(标准化为 1 X 10^6 个细胞/ml) 2D 和 3D 条件培养基中培养的 ASC 的 Flt-3 配体(图 4a)、IL-6(图 4b)和 SCF(图 4c)的水平。结果代表三个独立实验之一。图 4d 显示不同细胞蛋白的表达水平，根据用 iTRAQ 试剂标记之间比较的蛋白质样品的质谱法分析。蛋白样品采自在 2D(白条)和 3D(灰条)条件下生长的 ASC。该图代表两个重复实验中的一个。注意到 2D 和 3D 培养条件的条件培养基及细胞中某些蛋白的表达水平的差异。

图 5a-d 是描述源自胎盘的 3D-ASC 在体外分化为成骨细胞的能力的显微照片。源自人胎盘的 ASC 在导成骨分化的培养基(含有 10% FCS、100 nM 地塞米松、0.05 mM 抗坏血酸 2-磷酸盐、10 mM B-甘油磷酸盐的 DMEM)中培养 3 周时间。图 5a-b 显示表达钙化基质的细胞，根据由 AlizzarinRed S 染色所示。图 5c-d 显示不用导成骨分化的培养基处理的对照细胞，其保持成纤维细胞样表型并证明没有矿化。

图 6 是描述在移植后用化学疗法(连续 2 周腹膜内注射 25 mg/kg 白消安(busulfan))治疗 3.5 周的 NOD-SCID 小鼠骨髓(BM)中检测的人 CD45+细胞百分比的图表。将从源自脐带血的单核细胞纯化的 CD34+

细胞(100,000)单独移植(5只小鼠，a)，或与在2D条件下培养的0.5 X 10^6个源自胎盘的粘附细胞共同移植(2D-ASC；2只小鼠，b)，或与在pluriX™生物反应器中3D条件下培养的源自胎盘的粘附细胞(3D-ASC)共同移植(5只小鼠，c)。然后从小鼠大腿骨和胫骨收集BM。通过流式细胞术检测BM中的人细胞。通过让细胞与抗人CD45-FITC孵育来测定表达CD45的人细胞的百分比。注意到与单用HSC处理小鼠中的人类细胞的百分比(a)相比，与2D-ASC (b)以及与3D-ASC (c)共同移植的小鼠骨髓中的人细胞(hCD45+)的百分比更高。在经3D-ASC培养细胞处理的小鼠中观察到比经2D-ASC培养细胞处理的小鼠更高的移入，表明了经3D培养的ASC所特有的更高治疗优势。

图7a-b是仅用CD34+细胞移植的小鼠中的人移植CD45+细胞(图7a)与CD34+细胞加源自脂肪组织的ASC (图7b)比较的FACS分析。注意到与单用人CD34+处理的小鼠中的(7b-12%)相比，用源自脂肪组织的ASC共同移植的小鼠中的人造血群体(hCD45+)百分比(7a-29%)明显更高。

图8是描述在人脐带血单核细胞(CB)与等辐射量(3000 Rad)脐带血细胞(iCB)、源自人外周血单核细胞(PBMC)、2D培养的(2D)或3D培养的(3D)胎盘ASC、或PBMC和2D及3D培养的胎盘ASC的联合(PBMC+2D和PBMC+3D)之间进行混合淋巴细胞反应的条形图。CB细胞群大小由^{3}H-胸苷吸收(以CPM测量)来表示，在培养的最后18小时内测量。受刺激的CB细胞增殖升高说明较高的免疫应答水平。注意到与粘附细胞孵育的细胞显示出较低的免疫应答水平，特别是在与粘附细胞共同孵育时，对PBMC的CB免疫应答减少。每一反应作三个重复。

优选实施方案说明

本发明为新颖的细胞扩增方法和藉此产生的细胞和条件培养基用于干细胞相关治疗、干细胞移入和HSC支持的用途。

参考附图和随附说明可更好地理解本发明的原理和操作。

在详细解释至少一种本发明实施方案之前，应该理解本发明申请不限于在以下说明所阐述的或实施例例证中的细节。本发明能够有其它实施方案或以各种方式实践或实施。还应该理解，本文所用措辞和术语是为了说明目的，不应该认作限制。

在日益发展的医学界，为了临床和研究目的越来越需要干细胞，更具体地是基质干细胞(也称为“间充质干细胞”)。MSC 用于支持 HSC 移植和移入，也用于治疗越来越多种病症，例如心脏病、BM 缺乏症、神经元相关疾病和需要器官或组织移植的病症。

使用干细胞的障碍在于分离大量正常存在的干细胞或祖细胞群技术上困难，原因为：这些细胞在大部分组织中数量有限，在得到干细胞的方法中涉及不便之处和风险，并且用目前的收获方法伴随记忆 B 细胞和造血干细胞损失。从人胚胎获得细胞在已经存在的技术困难之上又增加了宗教和伦理方面的问题。

源自骨髓的干细胞的备选来源包括脂肪组织和胎盘。然而，目前尚无有效扩增来自这些组织的干细胞的方法。

在将本发明付诸于实践时，本发明人发现来自胎盘或脂肪组织的粘附细胞可在 3D 培养条件下有效繁殖。本发明人出乎意料之外地发现这样的细胞包含与 MSC 相似的功能特性，因此，这些细胞和由此产生的条件培养基可用作治疗目的，例如移植、组织再生和体内 HSC 支持。

如下文和以下实施例部分所述，本发明人能够在 3D 环境中扩增包含基质干细胞特性的源自脂肪组织和胎盘的粘附细胞。发现据此扩增的细胞在低温贮藏后有活力，这由粘附和重形成群体测定(repopulation assay)证明(参见实施例 1)。源自胎盘的粘附细胞的流式细胞术分析发现不同的标记表达型式(参见图 3a-b)。最重要的是，在 2D 或 3D 环境中繁殖的源自脂肪组织和胎盘的粘附细胞能够支持 HSC 移入(参见实施例 2)，其证实了本发明细胞作为基质干细胞在临床上的

用途。

因此，本发明一方面提供细胞扩增的方法。

所述方法包括在支持细胞扩增的三维(3D)培养条件下培养来自胎盘或脂肪组织的粘附细胞。

本文所用术语“扩增的”和“扩增”指基本上不分化地维持细胞及最终细胞生长，即使细胞群增加(例如至少到 2 倍)而不存在伴随所述增加的分化。

本文所用术语“维持的”和“维持”指基本上不分化的细胞更新，即基本上稳定细胞群而不存在伴随所述稳定的分化。

本文所用短语“粘附细胞”指具有贴壁依赖性的同质或异质细胞群，即细胞为了在体外生长需要粘附表面。

本文所用短语“脂肪组织”指包含脂肪细胞(adipocyte)的结缔组织。

本文所用术语“胎盘组织”指沿着子宫壁并在怀孕期间包裹胎儿的哺乳动物雌性器官的任何部分，胎盘和胎儿通过脐带连接。出生后胎盘剥离(称为产后胎盘)。

本文所用短语“三维培养条件”指将细胞置于与细胞生长相容同时使得细胞可以在多于一层上生长的条件。完全可将细胞在活生物体(或组织)中的原位环境理解为三维构造。细胞被其它细胞围绕。它们被固定在使得可建立各种局部微环境的纳米级细胞外基质纤维的复合网络中。它们的胞外配体不仅介导对基底膜的附着，而且还通向多种血管和淋巴管。氧、激素和营养素被运送到细胞而废物则被运出。本发明三维培养条件设计为模拟例如以下进一步作为例证的环境。

因此，本发明这方面的粘附细胞从脂肪组织或胎盘组织提取。

可从足月或早产胎盘得到胎盘细胞。优选一旦外出血就收集胎盘。优选在足以除去残留细胞的时间内灌注胎盘。本文所用术语“灌注(perfuse 或 perfusion)”指将液体倾注或使液体通过器官或组织的行为。胎盘组织可来自任何哺乳动物；最优选的胎盘组织是人的胎盘组

织。胎盘组织方便的来源是产后胎盘(例如 1-6 小时)，然而，胎盘组织或细胞的来源或分离胎盘组织的方法对于本发明而言并不重要。

源自胎盘的粘附细胞可来自胎盘的胎儿(即羊膜或胎盘内部部分，参见实施例 1)和母体(即基蜕膜和壁蜕膜)部分。在生理盐水缓冲液[例如磷酸盐缓冲盐水(PBS)或 Hank 缓冲液)中洗涤组织样品。通过用消化酶处理组织(参见下面)或/和切碎(mince)并用洗涤培养基经过尼龙滤器冲洗组织部分或通过轻轻吹打(Falcon, Becton, Dickinson, San Jose, CA)，制备单细胞悬浮液。

源自脂肪组织的粘附细胞可通过本领域技术人员已知的多种方法来分离。例如，这样的方法阐述于美国专利第 6,153,432 号。脂肪组织可源自网膜/内脏、乳腺、性腺或其它脂肪组织部位。优选脂肪组织来源为网膜脂肪组织。在人类中，脂肪组织通常通过吸脂来分离。

从脂肪组织分离粘附细胞可通过在如下条件处理组织得到：用消化酶(例如胶原酶、胰蛋白酶和/或分散酶；和/或有效浓度的透明质酸酶或 DNA 酶)；和乙二胺四乙酸(EDTA)；在 25-50℃的温度作用 10 分钟-3 小时。然后可以让细胞通过 20 微米-800 微米的尼龙或粗棉布网滤器。然后让细胞直接在培养基中或经过 Ficoll 或 Percoll 或其它颗粒梯度进行差速离心。细胞在 4-50℃温度以 100-3000 x g 速度离心 1 分钟-1 小时(参见美国专利第 7,078,230 号)。

除源自胎盘或脂肪组织的粘附细胞外，本发明还展望特征为基质干细胞表型(其将在下文进一步阐述)的来自其它细胞来源的粘附细胞的用途。可从其中提取粘附细胞的组织来源包括但不限于：脐带血、毛囊 [例如美国专利申请第 20060172304 号所述]、睾丸 [例如 Guan K. 等，Nature. 2006 Apr 27; 440(7088):1199-203 所述]、人嗅粘膜 [例如 Marshall, CT.等，Histol Histopathol. 2006 Jun:21(6):633-43 所述]、胚胎卵黄囊[例如 Geijsen N, Nature. 2004 Jan 8; 427(6970): 148-54 所述]和羊膜水[Pieternella 等(2004) Stem Cells. 22:1338-1345]，已知它们所有的都包括间充质干细胞。来自这些组织来源的粘附细胞可以通过

使这些细胞在粘附表面上培养分离，由此从其它细胞原始群中分离出粘附细胞。

不管是什么来源(例如胎盘或脂肪组织)，优选在无菌条件下提取细胞。一旦得到分离的细胞，就使其粘附到粘附材料(例如构成表面)上，藉此分离粘附细胞。这可在于 3D 培养条件中培养之前(参见实施例 1)或同时进行。

本文所用"粘附材料"指具有可让细胞保持在其表面的化学结构(例如荷有表面暴露基团)的人工合成、天然存在或其组合的无毒(即生物相容)材料。

可用于本发明这方面的粘附材料实例包括但不限于：聚酯、聚链烯、聚氟氯乙烯、聚氯乙烯、聚苯乙烯、聚砜、醋酸纤维素、玻璃纤维、陶瓷颗粒、matrigel、细胞外基质组分(例如纤维粘连蛋白、软骨粘连蛋白、层粘连蛋白)、胶原、聚 L 乳酸和惰性金属纤维。

用本领域熟知方法可实现进一步纯化或富集基质干细胞的步骤(例如通过用基质干细胞标记表达的 FACS，其在下文进一步阐述)。

用于本发明培养的基本培养基的非限制性实例包括极限必须培养基 Eagle、ADC-1、LPM(无牛血清白蛋白)、Fl0 (HAM)、F12 (HAM)、DCCM1、DCCM2、RPMI 1640、BGJ 培养基(进行和不进行 Fitton-Jackson 改良)、基本培养基 Eagle(BME-加入 Earle 碱盐)、Dulbecco 改良 Eagle 培养基(DMEM-无血清)、Yamane、IMEM-20、Glasgow 改良 Eagle 培养基(GMEM)、Leibovitz L-15 培养基、McCoy 5A 培养基、培养基 M199 (M199E-含 Earle 碱盐)、培养基 M199 (M 199H-含 Hank 碱盐)、极限必须培养基 Eagle (MEM-E-含 Earle 碱盐)、极限必须培养基 Eagle (MEM-H-含 Hank 碱盐)和极限必须培养基 Eagle (MEM-NAA，含非必须氨基酸)，在数目众多的其他培养基中包括：培养基 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。用于本发明的优选培养基为 DMEM。这些和其它有用的培养基购自 GIBCO, Grand Island, N. Y., USA 和 Biological Industries, Bet HaEmek, Israel 等等。大量这些培养基概述于酶学方法(Methods in Enzymology)第 LVIII 册,“细胞培养(Cell Culture)”,第 6272 页,William B. Jakoby 和 Ira H. Pastan 编辑,Academic 出版公司出版。

培养基可补充例如血清(例如牛或其它物种的胎血清或)和任选或备选以皮克/ml-毫克/ml 水平浓度的生长因子、细胞因子和激素(例如生长激素、促红细胞生成素、促血小板生成素、白介素 3、白介素 6、白介素 7、巨噬细胞集落刺激因子、c-kit 配体/干细胞因子、骨保护素配体、胰岛素、胰岛素样生长因子、表皮生长因子、成纤维细胞生长因子、神经生长因子、睫状神经营养因子、血小板源生长因子和骨形成蛋白)。

应进一步认识到可将另外组分加入培养基中。这样的组分可为抗生素、抗真菌素、白蛋白、氨基酸和本领域已知用于细胞培养的其它组分。此外,需要时可加入组分以提高分化过程(参见以下进一步阐述)。

一旦取得粘附细胞就可将其转移到三维环境(参见以下实施例部分的实施例 1)。但是应该了解,可在分离后立即将所述细胞转移到 3D 构造基质中(如上文所述)。

因此,使本发明这方面的粘附材料成形用于 3D 培养,藉此提供基本上增加对基质细胞粘附有用附着表面的生长基质,以便模拟组织(例如胎盘)的基础结构。

例如,对于 0.5 mm 高的生长基质而言,增加到至少 5-30 倍,其通过投影到生长基质基部来计算。所述增加到约 5-30 倍是对于每一单层而言,若使用许多所述层(无论是堆叠的还是由间隔物隔开或诸如此类),则 5-30 倍都适用于每一层如此结构。当基质以片(sheet)形式使用时,优选非编织纤维片或开孔发泡聚合物片,优选片厚度为约 50-1000 μm 或更厚,其提供供细胞进入、营养素进入和从片除掉废物用的足

够的多孔性。根据优选实施方案，所述孔具有 10 μm-100 μm 的有效直径。这样的片可从各种厚度的纤维制备，优选纤维厚度或纤维直径范围为约 0.5 μm-20 μm，更优选纤维直径范围为 10 μm-15 μm。

本发明结构可由提供空间稳定性和物理强度的多孔支撑片或筛网支撑，或甚至更好与其结合。

还可切割、冲孔或切碎这样的基质片，以提供投影面积约 0.2 mm-约 10 mm 且大约同样厚度(约 50 -1000 μm)的颗粒。

更多与用于使本发明付诸于实践的生长基质的制作、用途和/或优点阐述于美国专利第 5,168,08 5 号和特别是第 5,266,476 号中，它们都在此引作参考。

粘附表面可具有选自正方形、环形、圆形和十字形的形状。

对于大规模生产而言，优选在 3D 生物反应器中实现培养。

这样的物反应器实例包括但不限于推流式生物反应器、连续搅拌槽罐生物反应器和固定床生物反应器。

如实施例部分的实施例 1 所示，三维(3D)推流式生物反应器(如美国专利第 6911201 号所述)能够支持基质细胞的生长和长期维持。在该生物反应器中，基质细胞被接种到装于玻璃柱中由非编织纤维聚酯基质构成的 porrosive 载体上，藉此使大量细胞在相对小的体积内繁殖。

在推流式生物反应器中所用的基质可为片形式，为非编织纤维片或开孔发泡聚合物片，优选片厚度为约 50-1000 μm 或更厚，其提供供细胞进入、营养素进入和从片除掉废物用的足够的多孔性。

可与本发明使用的其它 3D 生物反应器包括但不限于连续搅拌罐生物反应器(其中使培养基连续进料到生物反应器中，将产物连续抽出，以维持反应器内的恒时稳态。带有纤维床篮的搅拌罐生物反应器可购自例如 New Brunswick Scientific 公司 Edison, NJ)、固定床生物反应器、气升式生物反应器(其中空气通常通入中央导管的底部，向上流动同时形成气泡，在柱顶分离废气)、带有 Polyactive 泡沫塑料的细胞接种灌注式生物反应器(如 Wendt, D.等，Biotechnol Bioeng 84: 205-

214, (2003)所述)、管状聚L乳酸(PLLA)多孔支架径向流灌注式生物反应器[如 Kitagawa 等， Biotechnology and Bioengineering 93(5): 947-954(2006)所述]。符合本发明的其它生物反应器阐述于美国专利第6,277,151号、第197,575号、第139,578号、第132,463号、第902,741号和第5,629,186号。

接种时优选细胞接种达到100,000-1,500,000个细胞/mm。

优选一旦达到至少约40%汇合、60%汇合或80%汇合就收获细胞，同时优选避免不受控制的分化和老化。

培养进行至少约2天、3天、5天、10天、20天、1个月或甚至更长。应该了解，在生物反应器中的培养可能延长这一时间。可能实现传代以增加细胞数目。

优选本发明粘附细胞包含至少一种"基质干细胞表型"。

本文所用"基质干细胞表型"指源自骨髓基质(即间充质)干细胞的典型结构或功能表型。

本文所用短语 "干细胞"指没有终末分化的细胞。

因此，例如，细胞可能具有纺锤形。作为备选或另外，细胞可表达基质干细胞象征的一种或一群(例如表面标记)标记。基质干细胞表面标记(阳性和阴性)实例包括但不限于 CD105+、CD29+、CD44+、CD73+、CD90+、CD34-、CD45-、CD80-、CD19-、CD5-、CD20-、CD11B-、CD14-、CD19-、CD79-、HLA-DR-和 FMC7-。其它基质干细胞标记包括但不限于酪氨酸羟化酶、巢蛋白和H-NF。

基质干细胞代表性功能表型实例包括但不限于 T 细胞抑制活性(不刺激T细胞而相反地对其抑制)、造血干细胞支持活性以及脂肪细胞、肝细胞、成骨细胞和神经细胞定向分化。

这些结构或功能特征中的任一项都可用于评定本发明细胞(参见以下实施例部分的实施例1-2)。

根据本发明教导产生的细胞群特征为独特的蛋白质表达谱，其如实施例部分的实施例1所示。因此，例如，根据本发明教导产生的胎

盘或脂肪组织粘附细胞能够表达和/或分泌高水平的所选择的因子。例如，这样的细胞表达或分泌 SCF、Flt-3、H2AF 或 ALDH X 比在 2D 培养中生长的胎盘或脂肪组织粘附细胞表达或分泌的高达至少 2、3、4、5、6、7、8、9、10、11 或优选 12 倍。另外或作为备选，本发明细胞群分泌或表达 IL-6、EEEF2、RCN2 或 CNN1 比在 2D 培养中生长的胎盘或脂肪组织粘附细胞表达或分泌的高达至少 2、3 或 5 倍水平。另外或作为备选，本发明细胞群特征为与 2D 培养的细胞比较各种其它蛋白表达水平较低。因此，例如，分泌或表达比在 2D 培养中生长的胎盘或脂肪组织粘附细胞表达或分泌的 Hnrph1、CD44 抗原同种型 2 前体、Papss2 或 rpL7a 的表达水平低至 0.6、0.5、0.25 或 0.125。

当进一步将本发明具体化到实践中时，本发明人认识到粘附基质细胞尤其是 3D-ASC 显示免疫抑制活性。如以下实施例部分的实施例 3 所示，在 MLR 测定中发现粘附基质细胞尤其是 3D-ASC 抑制人类脐带血单核细胞的免疫反应。因此，本发明细胞可包含可优先用于临床的生物活性(例如 T 细胞抑制活性、造血干细胞支持活性)。

当进一步将本发明具体化到实践中时，本发明人认识到本发明细胞的条件培养基可包含可优先用于临床的生物活性(例如 T 细胞抑制活性、造血干细胞支持活性)。

因此，本发明进一步展望条件培养基的收集和其照原样的或在用本领域熟知方法浓缩、富集或分馏的另外步骤后的应用。优选本发明条件培养基来自高生存力的细胞培养对数中期。

如上所述，本发明细胞和条件培养基特征为基质干细胞表型，因此可用于任何研究和可能受益于使用这样的细胞的任何临床应用中。

移植的 HSC 移入并启动血细胞生成依赖于复杂的过程，包括随趋化因子梯度穿过内皮细胞屏障归巢到骨髓并驻留在合适龛，同时在龛中的移植细胞、ECM 和间充质细胞之间建立物理接触。所有这些过程涉及一群复杂的分子，例如细胞因子、激素、类固醇、细胞外基质蛋白、生长因子、细胞间作用和黏附蛋白和基质蛋白。

已知移植后 2-3 天于受者 BM 中仅检测到 1-5%灌输的 HSC [Kerre 等，J Immunol. 167:3692-8.(2001)； Jetmore 等，Blood. 99:1585-93 (2002)]。

MSC 对造血移入的贡献部分在于抑制引起移植物抗宿主病的源自供者的 T 细胞产生[(GvHD，Charbord P.,和 Moore, K., Ann. N. Y. Acad. ScI 1044: 159-167(2005)； Maitra B 等，Bone Marrow Transplant. 33(6):597-604.(2004)； 美国专利第 6,010,696 号； 第 6555374 号]，部分在于提供造血干细胞(HSC)支持(即维持并帮助造血干细胞增殖、成熟和/或归巢)。

如以下实施例部分的实施例 2 所述，意想不到地发现源自胎盘和脂肪组织的粘附细胞即使在化疗后也支持 HSC 移入。

考虑到这些结果，可想象到本发明细胞或培养基可用于使用基质干细胞移植的任何临床应用中。

因此，本发明另一方面提供在需要治疗的对象中治疗可受益于基质干细胞移植的医学病症(例如病理、疾病、综合征)的方法。

本文所用术语“治疗”指抑制或阻滞病理进展和/或引起病理的减轻、消除或消退。本领域技术人员应该理解，可使用各种方法和测定来评估病理进展，同样地，可使用各种方法和测定来评估病理的减轻、消除或消退。术语“治疗”优选指减缓或缩小与癌病有关的症状。优选治疗治愈例如基本上消除与医学病症有关的症状。

本文所用“可受益于基质干细胞移植的医学病症”指可通过给予本发明细胞/培养基减缓的任何医学病症。

术语或短语“移植”、“细胞置换”或 “移入”在本文中可交替使用，指将本发明细胞引入到靶组织中。

本文所用术语“对象”指任何对象(例如哺乳动物)，优选人类对象。

本发明这方面的方法包括给予所述对象治疗有效量的本发明细胞或培养基(上述)，藉此治疗对象的可受益于基质干细胞移植的医学病症。

根据本发明这方面可给予的细胞包括上述可在二维或三维环境中培养的粘附细胞及其间充质和非间充质的部分或终末分化衍生物。

衍生来自本发明基质干细胞的特定谱系细胞的方法为本领域熟知。参见例如美国专利第 5,486,359 号、第 5,942,225 号、第 5,736,396 号、第 5,908,784 号和第 5,902,741 号。

细胞可为天然的，或遗传改良例如以便衍生的目的谱系(参见美国专利申请第 20030219423 号)。

细胞和培养基可为新鲜或冷冻(例如冷冻保存)制备的自体或非自体来源(即同种异体或异种)。

根据医学病症可给予所述对象另外的化学药物(例如免疫调节剂、化疗等)或细胞。

因此，为了改进干细胞移入(例如增加受者 BM 中的有存活力的 HSC 数目和最优化改进正常白血细胞计数)，例如可将本发明细胞/培养基在 HSC 移植之前、同时或之后给予。

优选 HSC 和基质细胞享有共同的 HLA 抗原。优选 HSC 和基质细胞来自单一个体。或者，HSC 和基质细胞来自不同个体。

术语或短语“移植”、“细胞置换”或 “移入”在本文中可交替使用，指将本发明细胞引入到靶组织中。所述细胞可源自受者或源自同种异体或异种供者。

因为当将非自体细胞给予身体时可能诱导免疫反应，所以已开发了几种方法来降低排斥非自体细胞的可能性。这些方法包括在移植前抑制受者免疫系统或将非自体细胞包被在免疫隔离(immunoisolating)半渗透膜中。

胶囊化(encapsulation)技术通常分为涉及小的球状载体的微胶囊和涉及较大的平板中空纤维膜的大胶囊(macroencapsulation) (Uludag, H.等，哺乳动物细胞胶囊化技术(Technology of mammalian cell encapsulation). Adv Drug Deliv Rev. 2000; 42:29-64)。

制备微胶囊的方法为本领域所述，包括例如如下公开的方法：Lu

MZ 等，用藻酸盐和 α-苯氧基亚肉桂基乙酰化聚(丙烯胺)的细胞胶囊化 (Cell encapsulation with alginate and alpha-phenoxycinnamylidene-acetylated poly (allylamine)). Biotechnol Bioeng. 2000, 70: 479-83; Chang TM 和 Prakash S，酶、细胞和遗传工程微生物的微胶囊封装方法(Procedures for microencapsulation of enzymes, cells and genetically engineered microorganisms). Mol Biotechnol. 2001, 17: 249-60; 和 Lu MZ 等，用光敏聚(丙烯胺 α-氰基亚肉桂基乙酸酯)胶囊封装细胞的新颖的方法(A novel cell encapsulation method using photosensitive poly (allylamine alpha-cyanocinnamylideneacetate))。J Microencapsul. 2000, 17: 245-51。

例如，通过将改良的胶原与甲基丙烯酸 2-羟乙酯(HEMA)、甲基丙烯酸(MAA)和甲基丙烯酸甲酯(MMA)三元聚合物壳混合来制备微胶囊，导致胶囊厚 2-5 μm。这样的微胶囊可进一步用另外的 2-5 μm 三元聚合物壳包被，以提供负电荷的光滑表面并使血浆蛋白吸收最小化 (Chia, S.M. 等，用作细胞胶囊化生物材料的多层微胶囊 (Multi-layered microcapsules for cell encapsulation Biomaterials). 2002 23: 849-56)。

其它微胶囊基于藻酸盐、海洋多糖(Sambanis, A，糖尿病治疗中的胶囊化胰岛 (Encapsulated islets in diabetes treatment). Technol. Ther.2003, 5: 665-8)或其衍生物。例如，可通过在氯化钙存在下聚阴离子藻酸钠和纤维素硫酸钠与聚阳离子亚甲基-胍共聚物盐酸盐之间的聚合电解质络合作用来制备微胶囊。

应该了解，当使用较小的胶囊时改进了细胞胶囊化。因此，当胶囊大小从 1 mm 减小到 400 μm 时，胶囊化细胞的质量控制、机械稳定性、分散特性和体外活性都得到改进(Canaple L.等，通过大小控制改进细胞胶囊化(Improving cell encapsulation through size control). J Biomater SciPolym 编辑，2002;13:783-96)。此外，发现孔径很好地控制在小至 7 nm 的、经处理(tailored)表面化学和精确微构造的纳米孔生物胶囊成功地为细胞免疫隔离出微环境(Williams D. 小即是美：医疗

装置中的微粒和纳米粒技术(Small is beautiful: microparticle and nanoparticle technology in medical devices). Med Device Technol. 1999, 10: 6-9; Desai, T.A.胰腺细胞胶囊化的微构造技术(Microfabrication technology for pancreatic cell encapsulation). Expert Opin Biol Ther. 2002, 2: 633-46)。

免疫抑制剂实例包括但不限于：氨甲蝶呤(methotrexate)、环磷酰胺(cyclophosphamide)、环孢霉素(cyclosporine)、环孢霉素A(cyclosporin A)、氯喹(chloroquine)、羟氯喹、柳氮磺吡啶(sulphasalazopyrine)、金盐、D-青霉胺(D-penicillamine)、来氟米特(leflunomide)、硫唑嘌呤(azathioprine)、阿那白滞素(anakinra)、英夫利昔单抗(infliximab) (REMICADE)、依那西普(etanercept)、TNF α阻断剂、靶向炎性细胞因子的生物药物、和非固醇类抗炎药物(NSAID)。NSAID 实例包括但不限于：乙酰水杨酸、水杨酸胆碱镁、二氟尼柳(diflunisal)、水杨酸镁、双水杨酸酯、水杨酸钠、双氯芬酸(diclofenac)、依托度酸、非诺洛芬(fenoprofen)、氟比洛芬(flurbiprofen)、吲哚美辛(indomethacin)、酮洛芬(ketoprofen)、酮咯酸、甲氯芬那酸酯、萘普生(naproxen)、萘丁美酮(nabumetone)、保泰松(phenylbutazone)、吡罗昔康(piroxicam)、舒林酸、托美丁(tolmetin)、对乙酰氨基酚(acetaminophen)、布洛芬(ibuprofen)、Cox-2 抑制剂和曲马多(tramadol)。

在本文所述任一方法中，可以给予细胞或培养基本身，或优选作为进一步包含药学上可接受载体的药物组合物部分来给予。

本文所用"药物组合物"指含其它化学组分(例如药学上合适的载体和赋形剂)的本文所述一种或多种化学缀合物的制剂。药物组合物目的是方便将化合物给予对象。

下文所用术语"药学上可接受的载体"指载体或稀释剂，其对对象不引起明显刺激，不消除所给予化合物的生物学活性和特性。载体的非限制性实例为丙二醇、盐水、乳液和有机溶剂与水的混合物。

本文所用术语"赋形剂"指加到药物组合物中以进一步方便施用化

合物的惰性物质。赋形剂的非限制性实例包括碳酸钙、磷酸钙、各种糖和各种类型淀粉、纤维素衍生物、明胶、植物油和聚乙二醇。

根据本发明优选实施方案，药学载体为水性盐溶液。

制备和给予药物的技术可在“雷明顿药物科学(Remington's Pharmaceutical Sciences)”，Mack 出版公司，Easton, PA，最新版本中找到，其在此引作参考。

可以全身方式给予药物组合物(如上文所述)。或者，可局部给予药物组合物，例如直接将药物组合物注射到患者的组织区。

可通过本领域熟知的方法来制备本发明药物组合物，例如借助于常规混合、溶解、成粒、制成糖锭剂、研粉、乳化、胶囊化、包埋或冻干的方法。

因此，可用一种或多种包含赋形剂和辅助剂的生理学上可接受的载体以常规方式来调配用于本发明的药物组合物，所述载体方便将活性成分加工成可在药学上使用的制剂。合适的制剂取决于所选择的给药途径。

对于注射而言，可以以水溶液来调配药物组合物的活性成分，优选以生理上可容的缓冲液例如 Hank 溶液、Ringer 溶液或生理盐水缓冲液。对于透过粘膜给药而言，制剂中使用适于待渗透的屏障的渗透剂。这样的渗透剂通常为本领域所知。

对于用于本发明方法中的任何制剂而言，可从体外和细胞培养测定中初始估计治疗有效量或剂量。优选在动物模型中调配剂量以达到所需的浓度或滴度。这样的资料可用于更准确地确定人类使用的剂量。

可通过体外标准药学方法在细胞培养或实验动物中测定本文所述活性成分的毒性和疗效。

从这些体外和细胞培养测定和动物研究中得到的数据可用于调配供人类使用的一系列剂量。剂量可视所用剂型和所用给药途径而变化。可通过个别医生考虑患者病症选择确切的制剂、给药途径和剂量

(参见例如 Fingl 等，1975，载于“治疗学的药理学基础(The Pharmacological Basis of Therapeutics)”，第 1 章第 1 页)。例如，可对帕金森患者的显示对治疗积极反应的运动功能进行症状监控。

对于注射而言，可以以水溶液调配药物组合物的活性成分，优选以生理上可容的缓冲液例如 Hank 溶液、Ringer 溶液或生理盐水缓冲液。

可个别调整剂量和给药间隔，以使活性成分水平足以通过移植的细胞来有效调节神经递质合成。达到理想效果所需的剂量将视个体特征和给药途径而定。检测试验可用于测定血浆浓度。

根据待治疗病症的严重程度和反应，可单次或多次给药，且疗程持续若干天到若干周，或达到减轻疾病状态。

当然，待给予的组合物的量将视正接受治疗的个体、病痛的严重程度、给药方式、主治医生的判断等等而定。给药剂量和时间应该对仔细连续监测的个体病情变化做出反应。例如，基于监测的指示，将给予被治疗的帕金森患者足以减轻疾病症状的细胞量。

本发明细胞经移植后优选在患病区域存活一段时间(例如至少 6 个月)，以便观察到治疗效果。

包括调配在药物可容载体中的本发明制剂在内的组合物可以经制备；置于合适容器中；并标上用于指定病症的治疗。

若有需要，本发明组合物可以存于包或分配装置(dipenser device)例如 FDA 核准的试剂盒中，其可含有含活性成分的一个或多个单位剂型。所述包可例如包含金属或塑料片，例如起泡包(blister pack)。所述包或分配装置可附有给药说明。包或分配装置还可提供与由管理药物生产、使用或销售的政府机构规定的形式的容器有关的公告，所述公告反映该机构批准的组合物形式或人类给药或兽用给药形式。这样的公告例如可以是由美国食品和药品管理局批准的处方药物标签或批准的产品插入物。

实施例

现在以下实施例和以非限制方式阐明本发明的上述说明一起构成参考。

本文所用术语和本发明所用实验方法通常包括分子、生化、微生物学和重组DNA技术。这些技术在文献中有详尽的解释。参见例如："分子克隆：实验手册(Molecular Cloning: A laboratory Manual)" Sambrook 等，(1989)；"分子生物学通用方案(Current Protocols in Molecular Biology)" 第I-III卷，Ausubel, R. M.编辑(1994)；Ausubel 等，"分子生物学通用方案(Current Protocols in Molecular Biology)", John Wiley 和 Sons, Baltimore, Maryland (1989); Perbal, "分子克隆实用指南(A Practical Guide to Molecular Cloning)", John Wiley & Sons, New York(1988)；Watson 等，"重组 DNA(Recombinant DNA)", Scientific American Books, New York; Birren 等(编辑)"基因组分子：实验手册丛书(Genome Analysis: A Laboratory Manual Series)", 第1-4卷，Cold Spring Harbor Laboratory Press, New York (1998)；美国专利第4,666,828号、第4,683,202号、第4,801,531号、第5,192,659号和第5,272,057号提出的方法；"细胞生物学：实验手册(Cell Biology: A Laboratory Handbook)"，第I-III卷 Cellis, J. E.编辑(1994)；"免疫学通用方案(Current Protocols in Immunology)"第I-III卷 Coligan J. E.编辑(1994)；Stites 等(编辑)，"基础和临床免疫学(Basic and Clinical Immunology)"(第8版), Appleton & Lange, Norwalk, CT(1994)；Mishell 和 Shiigi(编辑)，"细胞免疫学选择方法(Selected Methods in Cellular Immunology)", W. H. Freeman 和 Co., New York(1980)；广泛阐述于专利和科学文献中的有用的免疫测定，参见例如美国专利第3,791,932号、第3,839,153号、第3,850,752号、第3,850,578号、第3,853,987号、第3,867,517号、第3,879,262号、第3,901,654号、第3,935,074号、第3,984,533号、第3,996,345号、第4,034,074号、第4,098,876号、第4,879,219号、第5,011,771号和第5,281,521号；"寡核苷酸合

成(Oligonucleotide Synthesis)" Gait, M. J.编辑(1984); "核酸杂交(Nucleic Acid Hybridization)" Hames, B. D.,和 Higgins S. J.编辑(1985); "转录和翻译(Transcription and Translation)" Hames, B. D.,和 Higgins S. J.编辑(1984); "动物细胞培养(Animal Cell Culture)" Freshney, R. L 编辑(1986); "固定化细胞和酶(Immobilized Cells and Enzymes)" IRL Press,(1986); "分子克隆实行 指南(A Practical Guide to Molecular Cloning)" Perbal, B.,(1984)和 "酶学方法(Methods in Enzymology)" 第1-317卷, Academic Press; "PCR 方案: 方法和应用指南(PCR Protocols: A Guide To Methods and Applications)", Academic Press, San Diego, CA(1990); Marshak 等, "蛋白质纯化和鉴定策略-实验课手册(Strategies for Protein Purification and Characterization - A Laboratory Course Manual)" CSHL Press(1996); 以上所有如同本文完全提出一样在此引作参考。贯穿本文献提供了其它一般的参考资料。认为其中的方法为本领域熟知, 提供它们是为了方便读者。其中所包含的所有资料在此引作参考。

实施例 1

从骨髓、胎盘和脂肪组织生产和培养粘附基质细胞(ASC)

在含有 3D 载体的生物反应器系统中培养粘附细胞, 以生产特征为特定细胞标记表达谱的 3D-ASC 细胞。通过细胞计数测定生长效率。通过在分化培养基中培养来测定这些细胞的分化能力。

材料和试验方法

骨髓基质细胞 – 经过心脏直视手术或 BM 活检从血液学健康的供者吸出胸骨骨髓得到骨髓(BM)基质细胞。用 Hank 平衡盐溶液(HBSS; GIBCO BRL/Invitrogen, Gaithersburg MD)将骨髓吸出物稀释 3 倍, 进行 Ficoll-Hypaque (Robbins Scientific 公司, Sunnyvale, CA)密度梯度离心。此后收集骨髓单核细胞(<1.077 gm/cm3), 用 HBSS 洗涤 3 次, 重新悬浮于生长培养基[补充 10% FCS (GIBCO BRL)、10^{-4} M 巯基乙醇(Merck, White House Station, NJ)、青霉素-链霉素-制霉菌素混合

物(100 U/ml:100 ug/ml:1.25 un/ml; Beit Ha'Emek)、2 mM L-谷氨酰胺(Beit Ha'Emek)的 DMEM(Biological Industries, Beit Ha'emek, Israel)]。在组织培养培养瓶(Corning, Acton, MA)中于 37℃(5% CO_2)分开孵育来自各个供者的细胞且每周更换培养基。每 3-4 天用 0.25%胰蛋白酶-EDTA (Beit Ha'Emek)分裂(split)细胞。传 2-40 代后，当达到 60-80%汇合时，收集细胞用于分析或用于在生物反应器中培养。

源自胎盘的基质细胞 – 在无菌条件下切下足月分娩胎盘的里面部分(Bnei Zion 医学中心，Haifa, Israel)，用 Hank 缓冲液洗涤 3 次，于 37℃与 0.1%胶原酶(1mg / ml 组织; Sigma- Aldrich, St. Lewis, MO)孵育 3 小时。用吸管轻轻吹打，然后用补充 10% FCS、青霉素-链霉素-制霉菌素混合物(100 U/ml:100 ug/ml:1.25 un/ml)和 2 mM L-谷氨酰胺的 DMEM 洗涤悬浮的细胞，接种到 75 cm^2 培养瓶中并于 37℃在 5% CO_2 增湿条件下于组织培养培养箱中孵育。此后使细胞在塑料表面贴壁 72 小时，然后每 3 - 4 天换一次培养基。当达到 60-80%汇合(通常 10-12 天)时，用 0.25%胰蛋白酶-EDTA 从生长培养瓶分离细胞，并接种到新培养瓶中。此后收集培养的细胞用于分析或用于在生物反应器中培养。

源自脂肪组织的基质细胞 —从吸脂方法的人脂肪组织获得基质细胞(Rambam Haifa, Israel)。用等体积的 PBS 彻底洗涤脂肪组织，用胶原酶(20 mg/ml)于 37℃消化 30 分钟。然后用含有 10% FCS、青霉素-链霉素-制霉菌素混合物(100 U/ml:100 ug/ml:1.25 un/ml)和 L-谷氨酰胺的 DMEM 洗涤细胞，以 1200 rpm 在室温离心 10 分钟，用裂解液重新悬浮(1:10; Biological Industries, Beit Ha'emek, Israel, 以弃掉血细胞)，离心，用含有 10% FCS、青霉素-链霉素-制霉菌素混合物(100 U/ml:100 ug/ml:1.25 un/ml)和 L-谷氨酰胺的 DMEM 重新悬浮。然后以 3-10 x 10^7 个细胞/培养瓶将洗涤过的细胞接种到无菌组织培养基培养瓶。第二天用 PBS 洗涤细胞以除掉残留的 RBC 和死细胞。于 37℃在 5% CO_2 增湿条件下于组织培养培养箱中保持细胞。每 3-4 天更换培养

基。在 60-80%汇合时，用 0.25%胰蛋白酶-EDTA 从生长培养瓶分离细胞，并接种到新培养瓶中。传 2-40 代后，当达到 60-80%汇合时，收集细胞用于分析或用于在生物反应器中培养。

PluriX™ 推流式生物反应器 - 用由非编织纤维聚酯基质构成的 1-100 ml 包好的 3D porrosive 载体(直径 4 mm)装载 PluriX™推流式生物反应器(Pluristem, Haifa, Israel; 如图 1g 所述，也参见美国专利第 6,911,201 号)。这些载体使得可在相对小的体积内繁殖大量细胞数。由 Pluristem 设计并制造玻璃器皿。生物反应器在 37℃培养箱中保持，且流速由阀门(图 1g 中的 6a)和蠕动泵(图 1g 中的 9)调节并监控。生物反应器含有取样和注射点(图 1g 中的 4)，使得可连续接种细胞。从库(图 1g 中的 1)中供给 pH 6.7-7.4 的培养基。该库由含有不同比例的空气/CO_2/O_2 (由生物反应器中的细胞密度而定)的已过滤气体混合物(图 1g 中的 2、3)供给。O_2 的比例适合生物反应器出口的溶 O_2 水平，其由监控器(图 1g 中的 6)测定。经由硅胶管或扩散器(Degania Bet, Emek Hayarden, Israel)将气体混合物提供给所述库。培养基流经能够收集循环中的非粘附细胞的分离容器(图 1g 中的 7)。通过蠕动泵(图 1g 中的 9)使得培养基循环。生物反应器进一步配备有附加的取样点(图 1g 中的 10)和用于连续交换培养基的容器。

生产 3D-粘附基质细胞(3D-ASC) – 用胰蛋白酶处理按上所述培养的非汇合原代人粘附 2D 细胞培养物，用补充 10% FBS、青霉素-链霉素-制霉菌素混合物(100 U/ml:100 ug/ml:1.25 un/ml)和 2 mM L-谷氨酰胺的 DMEM 洗涤并重新悬浮，经由注射点接种(10^3-10^5个细胞/ml)到无菌推流式生物反应器(参见图 1g)的 3D 载体。接种前将 PBS-Ca-Mg (Biological Industries, Beit Ha'emek, Israel)填入生物反应器，高压灭菌(120℃, 30 分钟)，用含有 10%热灭活的胎牛血清和青霉素-链霉素-制霉菌素混合物(100 U/ml:100 ug/ml:1.25 un/ml)的 Dulbecco 生长培养基洗涤。流速保持在 0.1-5 ml/分钟。接种过程涉及停止循环 2- 48 小时，藉此使得细胞沉积在载体上。生物反应器保持在控制温度(37℃)

和 pH 条件(pH = 6.7-7.4)下；根据需要使用供给无菌空气和 CO_2 的培养箱。每周置换 2-3 次生长培养基。用新鲜的 DMEM 培养基每 4 小时-7 天替换循环培养基。在密度为 1 x 10^6-1 x 10^7 个细胞/ml(生长后 12-40 天)时，从生物反应器除掉总培养基体积，用 PBS 将生物反应器和载体洗涤 3-5 次。然后用胰蛋白酶-EDTA 从载体分离 3D-ASC 细胞；(Biological Industries, Beit Ha'emek, Israel；轻轻搅动 3-15 分钟，1-5 次)，此后重新悬浮于 DMEM 并冷冻保存。

3D-ASC 质量生物学测定 – 解冻并计数冻存的 3D-ASC 细胞。为评估细胞生存力，将 2 x 10^5 个细胞接种到 150 cm^2 组织培养培养瓶中，在接种后 7 天内评估其粘附能力和重形成群体(repopulation)。此后用荧光单克隆抗体流式细胞仪(Beckman Coulter, Fullerton, CA)分析 3D-ASC 膜标记表型。

用流式细胞术测定比较 3D 和 2D 培养的粘附细胞的细胞膜标记谱 - 将来自 2D 培养物和 3D 流动系统培养物中的 100,000 - 200,000 个粘附细胞悬浮在 5 ml 管中的 0.1 ml 培养基中，与饱和浓度的下述各种单克隆抗体(MAb)孵育(4℃，30 分钟，暗环境)：FITC-缀合的抗人 CD90 (Chemicon International 公司，Temecula, CA)、PE 缀合的抗人 CD73 (Bactlab Diagnostic, Ceasarea, Israel)、PE 缀合的抗人 CD105 (eBioscience, San Diego, CA)、FITC 缀合的抗人 CD29 (eBioscience, San Diego, CA)、Cy7-PE 缀合的抗人 CD45 (eBiosience)、PE 缀合的抗人 CD19 (IQProducts, Groningen, The Netherlands)、PE 缀合的抗人 CD14 Mab (IQProducts)、FITC 缀合的抗人 CD11b (IQProducts)和 PE 缀合的抗人 CD34 (IQProducts)或 FITC 缀合的抗人 HLA-DR Mab (IQProducts)。孵育后在含有 1%热灭活的 FCS 的冰冷 PBS 中洗涤细胞两次，重新悬浮于 500 µl 0.5%甲醛中，用 FC-500 流式细胞仪(Beckman Coulter, Fullerton, CA)分析。

用质谱分析比较 3D 和 2D 培养的粘附细胞的蛋白谱- 如上所述生产来自胎盘的源自 2D 和 3D 培养程序的 ASC。简言之，通过在增湿

5% CO_2 环境(atmosphere)下于 37℃在 175 cm^2 培养瓶中培养 0.3-0.75 x 10^6 个细胞 4 天直至达到 60 - 80%汇合来生产 2D 培养物。通过在含有 2000 个载体的生物反应器中接种 2-10 x 10^6 个细胞/克并培养 18 天来生产 3D 培养物。收获后洗涤细胞(x3)以除去所有血清，沉淀并冷冻。根据厂商的方案，从沉淀物分离蛋白质[使用 Tri 试剂盒(Sigma, Saint Louis, USA)，用胰蛋白酶消化并用 iTRAQ 试剂标记(Applied Biosciences, Foster City, CA)]。简言之，iTRAQ 试剂为非聚合物的同量异位标记试剂。用四种同量异位的同位素编码标记中的一种经由其 N 末端和/或赖氨酸侧链标记每一样品中的肽。混合四个经标记的样品，用质谱法分析肽。在肽片段上的每种标记释放独特的质量报告离子，因此，四种报告离子的比值给出样品中特定肽的相对丰度(资料见: http: //docs.appliedbiosystems.com/pebiodocs/00113379.pdf)。

在 Smoler 蛋白质组中心(department of Biology, Technion, Haifa, Israel)用 LC-MS/MS 在 QTOF-Premier(Waters, San Francisco, CA)上实施源自胎盘的 ASC 的 2D 培养物对 3D 培养物的蛋白质组分析，且通过 Pep-Miner 软件[Beer, I.等，Proteomics, 4, 950-60(2004)]针对 nr 数据库的人类部分进行鉴别和分析。被分析的蛋白质有：核内不均一核糖核蛋白 H1 (Hnrph1，GeneBank 登录号 NP_005511)、H2A 组蛋白家族(H2AF, GeneBank 登录号 NP_034566.1)、真核细胞翻译延伸因子 2 (EEEF2, GeneBank 登录号 NP_031933.1)、网钙结合蛋白 3、EF-手形钙结合结构域(RCN2, GeneBank 登录号 NP 065701)、CD44 抗原同种型 2 前体(GeneBank 登录号 NP_001001389、钙调理蛋白蛋白 1 碱性平滑肌(CNN1, GeneBank 登录号 NP_001290)、3 磷酸腺苷 5 磷酰硫酸合成酶 2 同种型 a (Papss2, GeneBank 登录号 NP_004661)、核糖体蛋白 L7a (rpL7a, GeneBank 登录号 NP_000963)和醛脱氢酶 X (ALDH X, GeneBank 登录号 P47738)。每一实验进行 2 次。因为分析的特性，每一蛋白质按照出现在样品中的肽数目来分析(在每一次分析中蛋白质出现 2-20 次)。

用 ELISA 比较 3D 和 2D 培养的粘附细胞分泌的蛋白质- 如上所述生产来自胎盘的源自 2D 和 3D 培养方法的 ASC，且 3D 培养持续 24 天。此后收集条件培养基，在三个独立实验中，用 ELISA (R&D 系统, Minneapolis, MN)对 Flt-3 配体、IL-6、促血小板生成素(TPO)和干细胞因子(SCF)进行分析。结果标准化为 1 x 10^6 个细胞/ml。

成骨细胞分化培养基 - 通过在由补充 10% FCS、100 nM 地塞米松、0.05 mM 抗坏血酸 2-磷酸盐、10 mM B-甘油磷酸盐的 DMEM 组成的成骨细胞分化培养基中培养细胞 3 周来评估成骨细胞分化。由 Alizzarin Red S 染色显示钙化基质，由碱性磷酸酶测定试剂盒(所有试剂来自 Sigma- Aldrich, St. Lewis, MO)检测碱性磷酸酶。

结果

PluriX™生物反应器系统创造了生理样微环境

为了给粘附细胞提供有效的培养条件，用 PluriX 生物反应器 (Pluristem, Haifa, Israel; 载体在图 1g 中阐明，在接种前显示于图 1b 中)人工创造生理样环境(图 1a 中所述)。如图 1c-f 所示，骨髓产生的 3D-ASC 细胞在 3D 基质上成功培养并在接种后扩增 20 天(图 1b-c，分别放大 x 150 和 250)和 40 天(图 lc-d，分别放大 x 350 和 500)。

生长在 PluriX 生物反应器系统中的细胞显著扩增 - 不同生产批次的源自胎盘的 3D- ASC 细胞在 PluriX 生物反应器系统中生长。接种密度为 13,300 个细胞/载体(至总量为 2 x 10^6 个细胞)。接种 14 天后，细胞密度增加到 15 倍，达到大约 200,000 个细胞/载体(图 2)，或者说在 150 载体的生物反应器中 30 x 10^6。在不同的实验中，将细胞以 1.5 x 10^4 细胞/ml 密度接种到生物反应器，接种 30 天后，载体含有超过 50 倍的更大的细胞数量，即大约 0.5 x 10^6 个细胞/载体或 0.5 x 10^7 个细胞/ml。在各种水平生长柱的载体上的细胞密度一致，说明氧和营养素均一传递到细胞。由此证明 3D 培养系统为高密度间充质细胞培养物的生长和长期维持提供支持条件，所述细胞培养物可以有效生长到足以用于支持移入和成功移植目的的量。

3D-ASC 显示独特的膜标记特征 - 为了确定可溶性分子分泌谱及蛋白质产生(通过模拟骨环境的 3D 培养方法来实施)的差异，进行了 FAC 分析。如图 3a 所示，细胞标记的 FACS 分析描述的是，3D-ASC 表现出与在 2D 条件中生长的粘附细胞不同的标记表达型式。与 3D 培养的细胞相比，2D 培养的细胞表达明显更高水平的阳性膜标记 CD90、CD105、CD73 和 CD29 膜标记。例如，3D 培养的细胞显示 56% 的 CD105 表达，对比 2D 培养的细胞为 87%。2D 和 3D 二者胎盘培养物的 ASC 都不表达任何造血膜标记(图 3b)。

3D-ASC 显示独特的可溶性因子谱 - 造血龛包括产生丰富细胞因子、趋化因子和生长因子的支持细胞。为了进一步确定 2D 和 3D 培养的 ASC 之间的差别，通过 ELISA 得到 2D 和 3D ASC 培养物条件培养基中四种主要的造血分泌蛋白谱。图 4a-c 显示生长在 3D 条件的细胞产生含更高水平的 Flt-3 配体(图 4a)、IL-60 (图 4b)和 SCF (图 4c)的条件培养基，而在 2D 培养物的条件培养基中检测到较低水平的 IL-6 和接近零水平的 Flt-3 配体和 SCF。在两种培养物中促血小板生成素(TPO)产量都很低并不相上下。

3D-ASC 在质谱分析中显示独特的蛋白谱 – 为了进一步确定 2D 和 3D 培养的 ASC 之间的差异，用质谱分析这些细胞的蛋白谱。图 4d 表示 2D 和 3D 培养的 ASC 展现出明显不同的蛋白质表达谱。如下表 1 所示，3D 培养的细胞表现出更高的 H2AF 和 ALDH X 表达水平(分别高出超过 9 和 12 倍)和更高的 EEEF2、RCN2 和 CNN1 蛋白水平(分别为大约 3、2.5 和 2 倍)。另外，3D 培养的细胞展现大约一半的蛋白 Hnrph1 和 CD44 抗原同种型 2 前体表达水平和大约三分之一的 Papss2 和 rpL7a 表达水平。

表 1

蛋白质	蛋白水平(相对于 iTRAQ 报告基团)			
	3D 培养的 ASC		2D 培养的 ASC	
	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 抗原同种型 2 前体	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-ASC 具有分化为成骨细胞的能力 – 为了进一步表示 3D-ASC 的特征，在成骨细胞分化培养基中将细胞培养 3 周。此后实施钙沉淀。分化的细胞显示产生了钙(图 5a-b 中的红色所描述)，反之对照细胞维持成纤维细胞样表型并表明没有矿化(图 5c-d)。这些结果表明源自胎盘的 3D-ASC 具有体外分化为成骨细胞的能力。

实施例 2

源自胎盘的 3D-ASC 改进 HSC 移入的能力评估

通过在亚致死辐射或化疗预处理的免疫缺陷 NOD-SCID 小鼠中检测人造血细胞(hCD45+)水平，来评估 3D-ASC 支持 HSC 移入。

材料和实验方法

分离 CD34+细胞 – 在分娩期间于无菌条件下取脐带血样品(Bnei Zion Medical Center, Haifa, Israel)，用 Lymphoprep (Axis-Shield PoC As, Oslo, Norway)密度梯度离心分层单核细胞并冷冻保存。洗涤解冻的单

核细胞并用抗 CD34 抗体孵育，用 midi MACS (Miltenyl Biotech, Bergish Gladbach, Germany)分离。将来自一个以上样品的细胞合并达到所需的量(50,000-100,000 个细胞)。

在经辐射小鼠中检测移植的细胞 - 在无菌开放系统笼中供养 7 周龄雄性和雌性 NOD-SCID 小鼠(NOD-CB 17-Prkdcscid/J; Harlan/ Weizmann Inst., Rehovot Israel)，给予无菌饮食和高压灭菌的酸性水。亚致死(350 cGy)辐射小鼠，此后(辐射后 48 小时)通过静脉注射至尾静脉，移植 50,000-100,000 个 $hCD34^+$细胞，加上或不加另外的源自胎盘或脂肪组织的 ASC (0.5×10^6 - 1×10^6) (每组 3-7 只小鼠)。移植 4-6 周后，脱臼处死小鼠，用 FACS 缓冲液(50 ml PBS, 5 ml FBS, 0.5 ml 叠氮钠 5%)从大腿骨和胫骨两处冲洗收集 BM。通过流式细胞术检测小鼠 BM 中的人细胞，通过使细胞与抗人 CD45-FITC (IQ Products, Groningen, The Netherlands)孵育，产生在处理的 NOD-SCID 小鼠中表达人和鼠 CD45 造血细胞标记的细胞百分比。明确的人移入最低阈限指定为 0.5%。

化疗处理的小鼠中移植细胞的检测 –如上述用于辐射小鼠的一样供养的 6.5 周龄雄性 NOD-SCID 小鼠(NOD.CB17/JhkiHsd-scid; Harlan, Rehovot Israel)接受白消安(25 mg/kg-连续 2 天)腹膜内注射。第二次注射白消安 2 天后，单用 CD34+细胞或与从胎盘产生的 0.5×10^6 个 ASC 一起注射小鼠。移植后 3.5 周，处死小鼠，如上文对辐射小鼠所述测定人造血细胞的存在情况。

结果

3D-ASC 改进辐射小鼠的 HSC 移入- 在经辐射 NOD-SCID 小鼠中共同移植人 CD34+造血细胞和源自胎盘或脂肪组织的 3D-ASC。在共同移植之后 4 周评估移入效率，并与单用 HSC 移植的小鼠比较。如表 2 和图 6 所示，与单用 UCB CD34+细胞处理的小鼠比较，3D- ASC 和 UCB CD34+细胞共同移植导致移入率高相当多及受者小鼠 BM 中人细胞的水平高相当多。

表 2

移植的细胞	平均 h-CD45	STDEV
CD34	3.8	7.9
CD34+来自胎盘的 3D-ASC	5.1	12.2
CD34+来自脂肪组织的 3D-ASC	8.7	9.6

3D-ASC 改进化疗小鼠的 HSC 移入 -将人 CD34+造血细胞与源自胎盘的 500,000-2D-ASC 或 3D-ASC 共同移植到经化疗预处理的 NOD-SCID 小鼠中。

在共同移植之后 3.5 周评估移入效率，并与单用 HSC 移植的小鼠比较。如表 3 所示，与单用 UCB CD34+细胞相比，ASC 和 UCB CD34+细胞共同移植导致受者小鼠 BM 中的移入水平更高。此外，如表 3 所示，用在 PluriX 生物反应器系统中生长的源自胎盘的粘附细胞(3D-ASC)共同移植的小鼠，比在常规静态 2D 培养条件(培养瓶)中生长的来自同样供者的细胞共同移植的小鼠的平均移入水平更高。

表 3

移植的细胞	平均 h-CD45	STDEV
CD34	0.9	1.1
CD34+来自胎盘的常规 2D 培养物	3.5	0.2
CD34+来自胎盘的 3D-ASC	6.0	7.9

图 7a-b 中所示的 FACS 分析结果证明 ASC 与 hHSC (图 7b)共同移植的优势，以及在 HSC 移植后 ASC 改进造血系统恢复的能力。

这些结果综合起来表明，ASC 可在 HSC 移植(自体或同种异体)之后作为支持细胞改进造血恢复。3D-ASC 在 HSC 移植之后提高造血干细胞和/或祖细胞移入的能力可来自 3D-ASC 分泌可改进移植细胞的归巢、自我更新和增殖能力的支持 HSC 的细胞因子的能力，或来自那些细胞重建可移植的 HSC 归巢和增殖所需的受损造血微环境的能

力。

实施例 3

通过 2D 和 3D 培养的 ASC 抑制淋巴细胞应答

在 MLR 分析中发现粘附基质细胞尤其是 3D-ASC 抑制人类脐带血单核细胞的免疫反应。

材料和实验方法

混合淋巴细胞反应(MLR)测定 – 通过 MLR 测定法测定源自 2D 和 3D 培养方法的从胎盘产生的 ASC 的免疫抑制和免疫赦免(immunoprivilege)特性，MLR 测定测量位于 HLA 基因座的组织相容性，根据在 答应细胞(增生)和刺激细胞(不能增生)的混合培养中不相容淋巴细胞的增殖率来实现。使用人脐带血(CB)单核细胞(2×10^5)作为应答细胞，其通过与等量(10^5)辐射(3000Rad)过的源自人外周血单核细胞(PBMC)、或者与 2D 或 3D 培养的由胎盘产生的粘附细胞或粘附细胞和 PBMC 组合的共同培养来刺激。每一测定重复三次。细胞在 96-孔板中的 RPMI 1640 培养基(含 20% FBS 在增湿的 5% CO_2 环境于 37℃)中共同培养 4 天。在培养的最后 18 小时用 1μC ^{3}H-胸苷脉冲该板。然后经玻璃纤维滤器收集细胞，用闪烁计数器定量胸苷吸收。

结果

图 8 显示 CB 细胞的免疫应答，这根据当用 PBMC 刺激时这些细胞的增殖增加来表现，在不受任何理论的束缚下这很可能与响应 HLA 的不相容性的 T 细胞增殖有关。然而，当与本发明粘附细胞孵育时，这些细胞显示出相当低的免疫应答水平。此外，当与这些粘附细胞共同孵育时，CB 对 PBMC 的免疫应答基本降低了。因此，以与 MSC 相似的方式，发现 ASC 具有潜在的降低供者细胞的 T 细胞增殖(典型的是 GvHD)的能力。尽管 2D 和 3D 两种培养物都降低了淋巴细胞免疫应答，但与上文所述的 3D-ASC 的其它优点一致，3D ASC 的免疫抑制更强。

应该了解，为清楚起见，在各别实施方案的上下文中所阐述的本发明某些特征也可在单个实施方案中组合提供。反之，为简短起见，在单个实施方案上下文中所阐述的本发明的各种特征，也可分开或以任何合适的亚组合提供。

尽管本发明联合其具体实施方案已得到阐述，但显然很多备选、修改和变更对于本领域技术人员来说将显而易见。因此，意欲包括落在附加权利要求的精神和广泛范围内的所有这样的备选、修改和变化。本说明书所提到的所有出版物、专利和专利申请和 GenBank 登记号以其整体在此引作本说明书的参考，其程度如同明确并单独地指出将每一单独出版物、专利或专利申请或登记号在此引作参考一样。另外，本申请中的任何参考的附加、引用或认同不应该被理解为承认这样的参考可用作本发明的现有技术。

密质骨 PluriX™ 载体*150 20 天*150 20 天*250 40 天*350 40 天*500

图 1a 图 1b 图 1c 图 1d 图 1e 图 1f

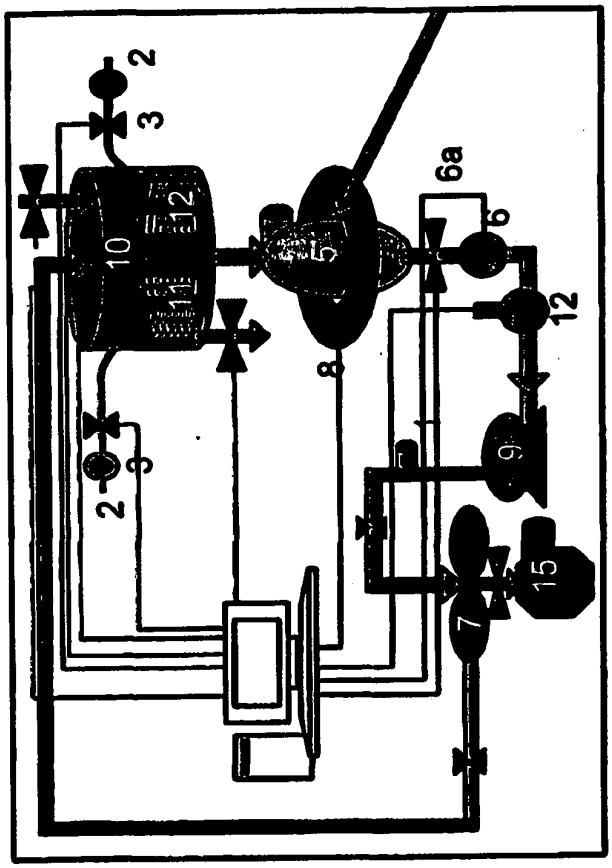


图 1g

图 2

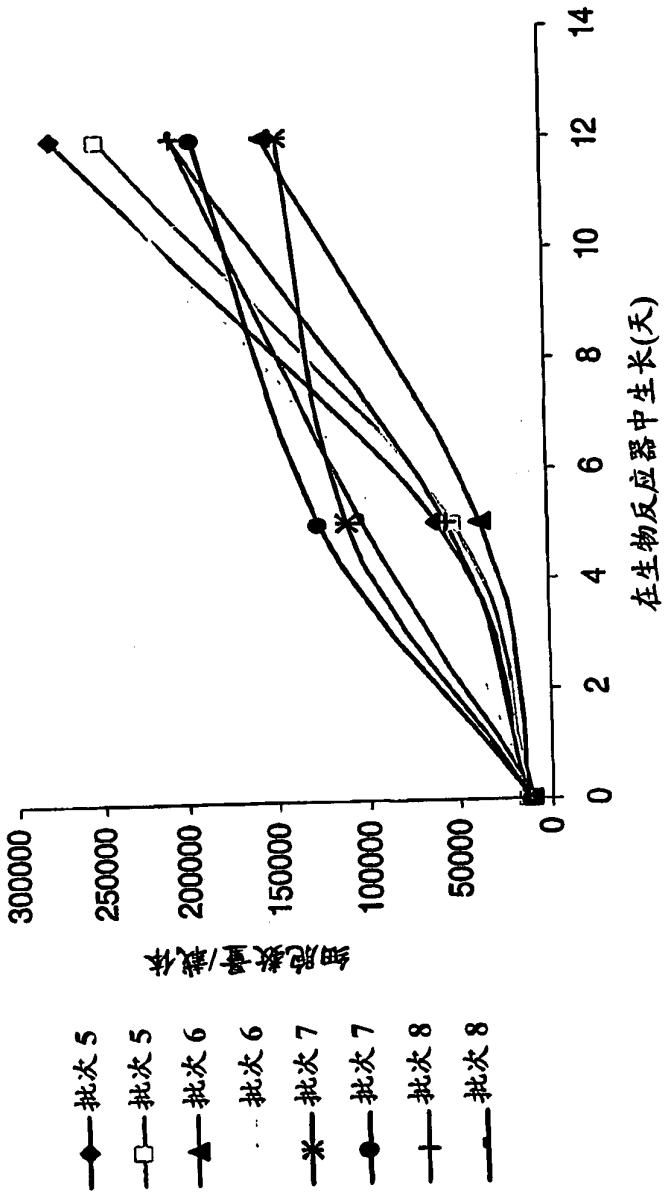
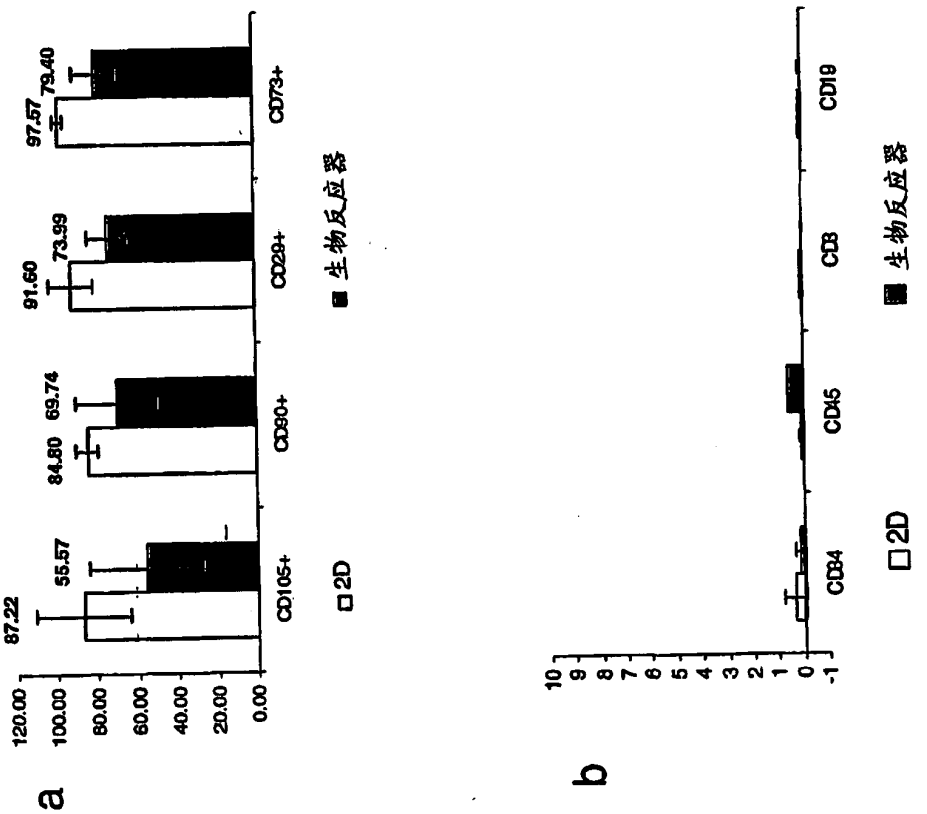

300000
250000
200000
150000
100000
50000
0
细胞数量/载体
0
2
4
6
8
10
12
14
在生物反应器中生长(天)
批次 5
批次 5
批次 6
批次 6
批次 7
批次 7
批次 8
批次 8

图 3a-b


a
120.00
100.00
80.00
60.00
40.00
20.00
0.00
87.22
55.57
84.80
69.74
91.60
73.99
97.57
79.40
CD105+
CD90+
CD29+
CD73+
□2D
■生物反应器
b
10
9
8
7
6
5
4
3
2
1
0
-1
CD34
CD45
CD8
CD19
□2D
■生物反应器

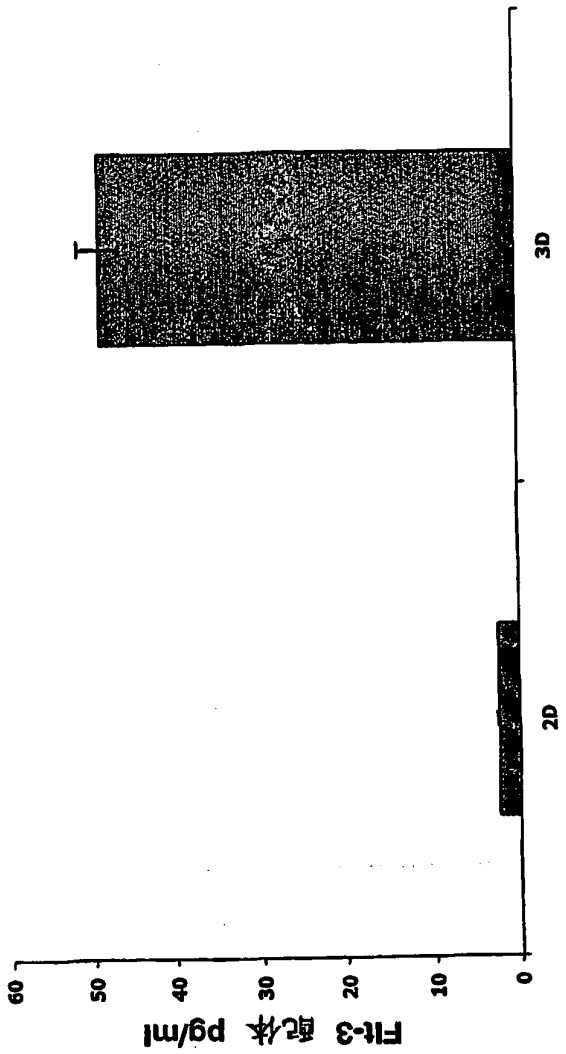


图 4a

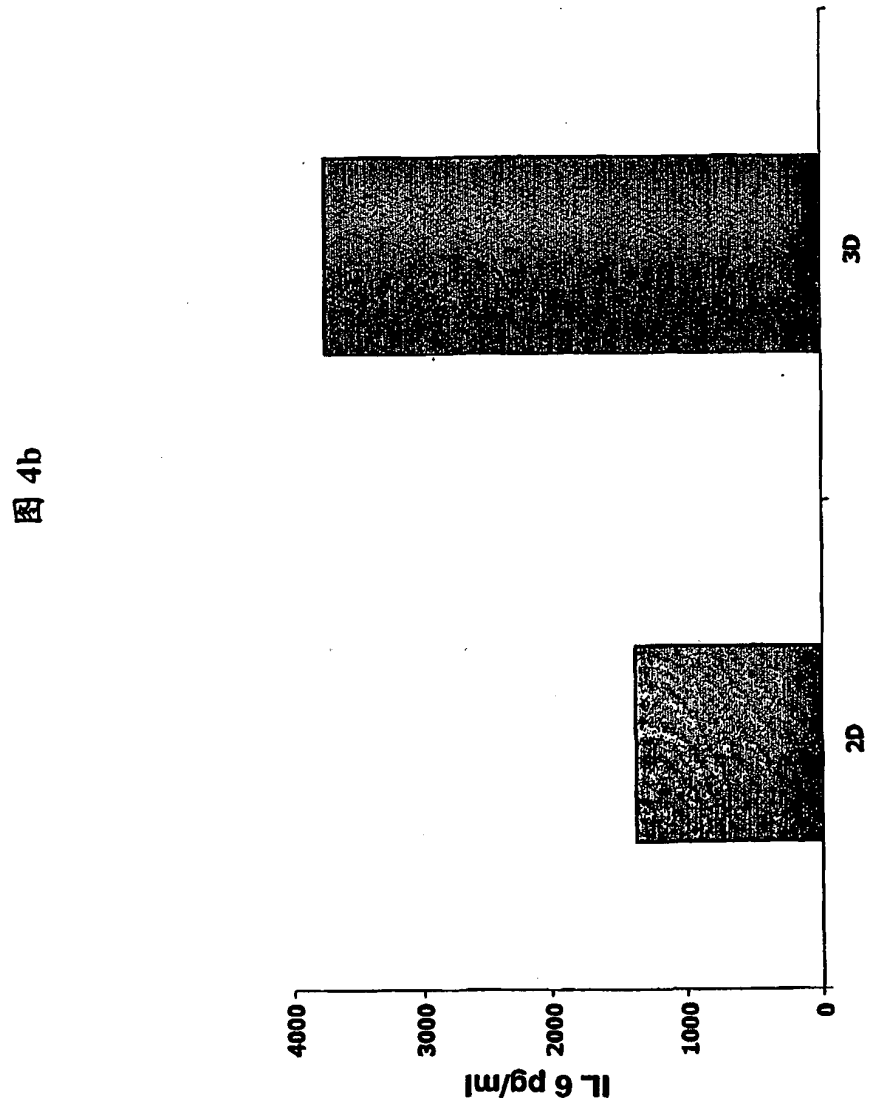


图 4b

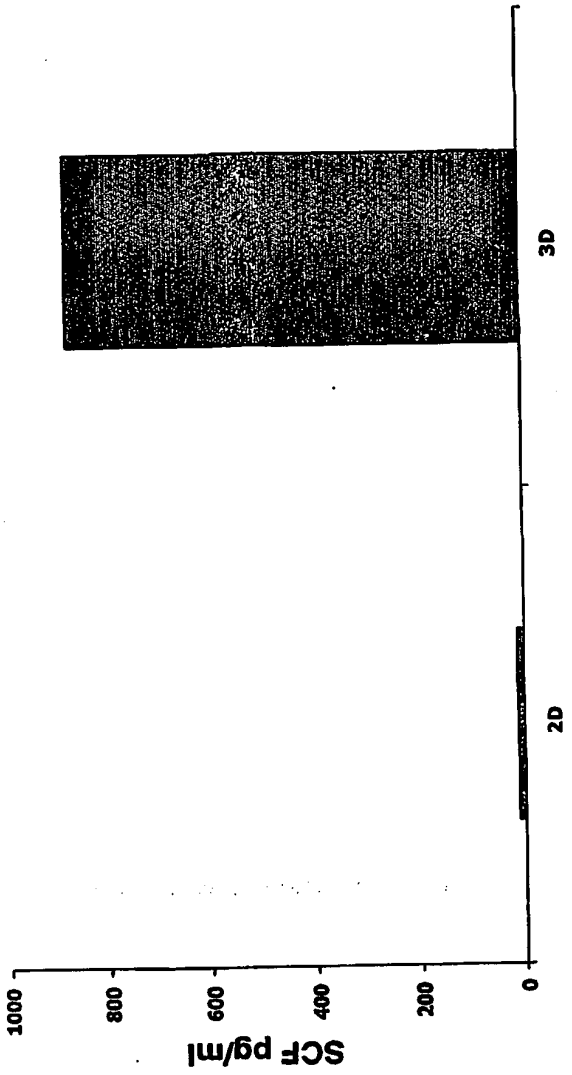


图 4c

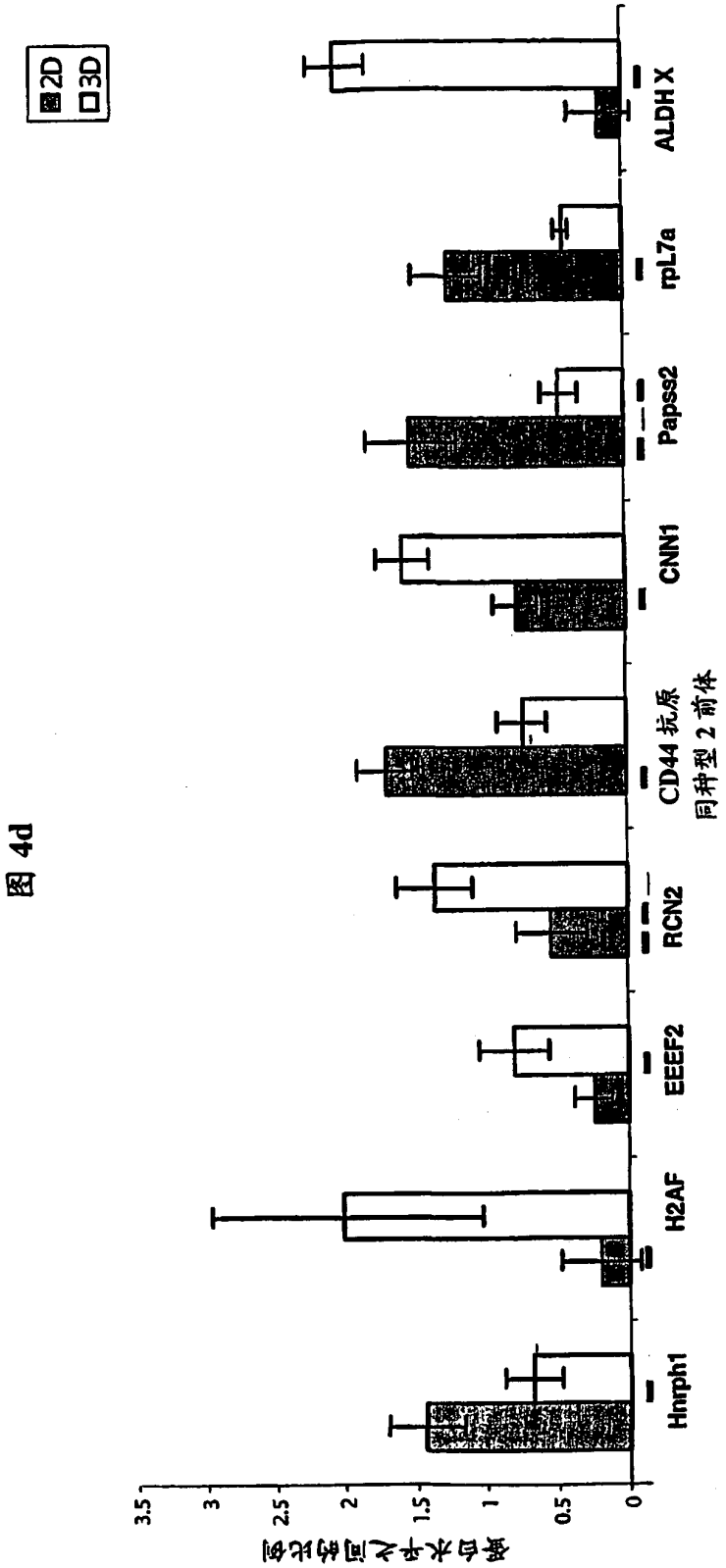

2D
3D
蛋白水平之间的比例
3.5
3
2.5
2
1.5
1
0.5
0
Hnrph1
H2AF
EEEF2
RCN2
CD44 抗原
同种型 2 前体
CNN1
Papss2
rpL7a
ALDH X

图 4d

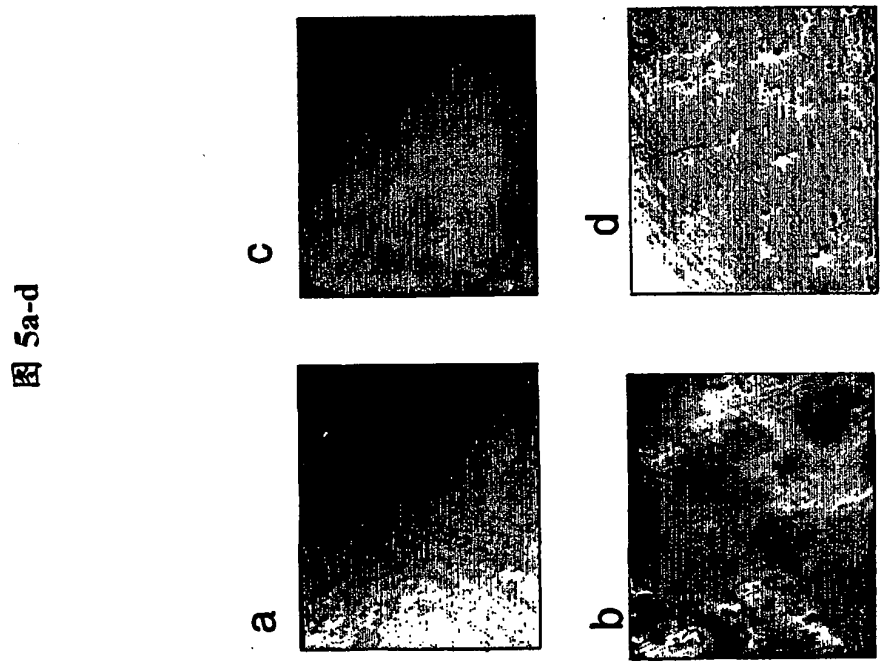


图 5a-d

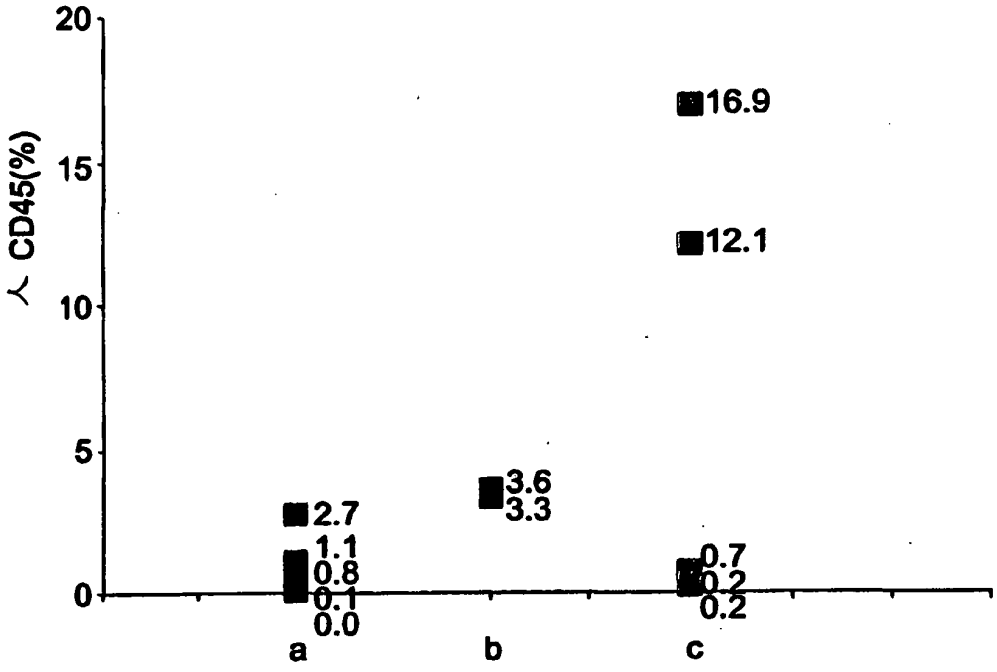


图 6

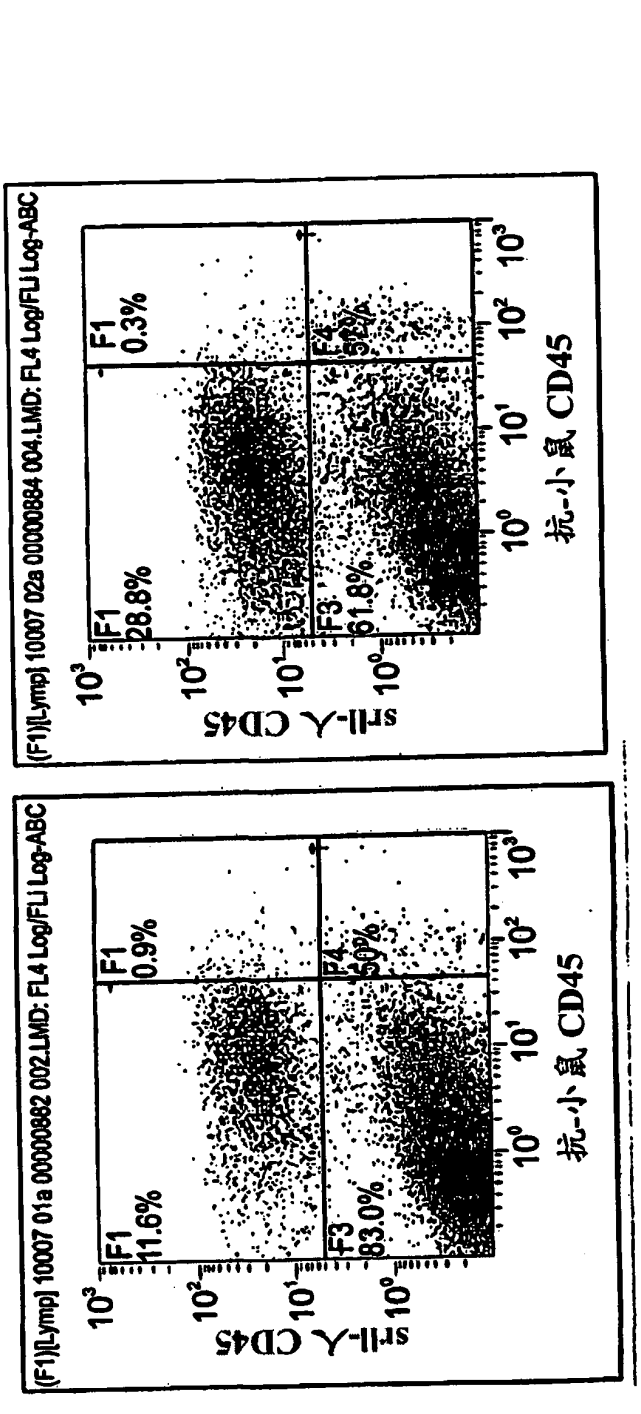


图 7a

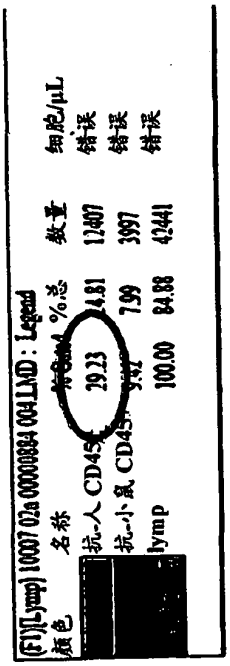
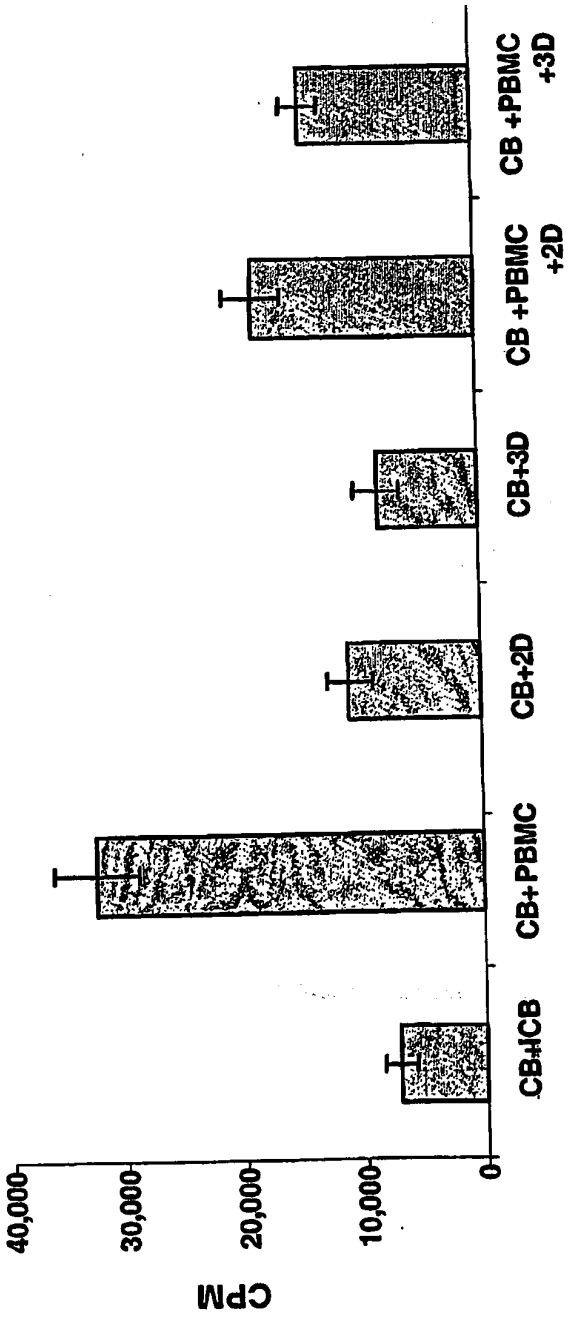


图 7b

人 CD45+ (%)
小鼠 CD45+ (%)

图 8


CPM
40,000
30,000
20,000
10,000
0
CB#CB
CB+PBMC
CB+2D
CB+3D
CB +PBMC +2D
CB +PBMC +3D