

**MORTAL PLURIPOTENT STEM CELLS****CROSS-REFERENCE**

**[0001]** This application claims the benefit of U.S. Provisional Application No. 63/020,247, filed May 5, 2020, which application is incorporated herein by reference in its entirety.

**BACKGROUND**

**[0002]** There exists a need for novel stem cells for treating various diseases or conditions, as an alternative to overcome certain shortcomings of existing embryonic stem cells and iPS cells.

**INCORPORATION BY REFERENCE**

**[0003]** All publications, patents, and patent applications herein are incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. In the event of a conflict between a term herein and a term in an incorporated reference, the term herein controls.

**BRIEF SUMMARY**

**[0004]** The inventive embodiments provided in this Brief Summary of the Invention are meant to be illustrative only and to provide an overview of selective embodiments disclosed herein. The Brief Summary of the Invention, being illustrative and selective, does not limit the scope of any claim, does not provide the entire scope of inventive embodiments disclosed or contemplated herein, and should not be construed as limiting or constraining the scope of this disclosure or any claimed inventive embodiment.

**[0005]** In some of many aspects, disclosed herein is a population of mortal pluripotent stem cells (MPSCs), wherein the population of MPSCs express HLA-G and insulin, and wherein the population of MPSCs are capable of reaching up to at least 89 population doublings within 90 days from a start of culturing the MPSCs. In some instances, the population of MPSCs are capable of reaching from about 89 to about 100 population doublings within 90 days from a start of culturing the MPSCs. In some instances, the population of MPSCs are capable of reaching from about 25 to about 30 population doublings within about 12 days, from about 50 to about 55 population doublings within about 30 days, and/or from about 75 to about 80 population doublings within about 63 days, from a start of culturing the MPSCs. In some instances, the population of MPSCs are capable of doubling in from about 22 to about 27 hours, for example about 25 hours. In some aspects, disclosed herein is a population of mortal pluripotent stem cells (MPSCs), wherein the population of MPSCs express HLA-G and insulin, and wherein the population of MPSCs are free from a pathogen.

**[0006]** In some cases, the MPSCs disclosed herein are free from a pathogen. In some instances, the MPSCs are free from a bacterium. In some instances, the MPSCs are free from a virus, for example a cytomegalovirus. In some instances, the MPSCs are free from a pathogen selected from the group consisting of EBV (Epstein-Barr virus), HAdV (human adenovirus), HCMV (human cytomegalovirus), a Hepatitis virus (*e.g.*, Hepatitis A, Hepatitis B, and/or Hepatitis C), a human herpes virus (*e.g.*, HHV 6 (human herpes virus 6) and/or HHV 8 (human herpes virus 8)), a human immunodeficiency virus (*e.g.*, HIV1 (human immunodeficiency virus 1), HIV2 (human immunodeficiency virus 2)), a human papillomavirus (*e.g.*, HPV16, HPV18, *etc.*), a herpes simplex virus (*e.g.*, HSV 1 (herpes simplex 1), HSV 2 (herpes simplex 2), *etc.*), a human T-lymphotropic virus (*e.g.*, HTLV 1 (human T-lymphotropic virus 1), HTLV 2 (human T-lymphotropic virus 2), *etc.*), VZV (varicella virus), *Corynebacterium bovis*, *Corynebacterium sp.* (HAC2), a Hantavirus (*e.g.*, Hantaan, Seoul, or Sin Nombre), a lymphocytic choriomeningitis virus (LCMV), a *Mycoplasma sp.*, *Treponema pallidum*, and any combination thereof.

**[0007]** In some instances, the MPSCs disclosed herein, or a population comprising the MPSCs, further express one or more proteins of beta Human chorionic gonadotropin (b-HCG), heat shock protein 90 (HSP90), Caudal Type Homeobox 2 (CDX2), Fibroblast growth factor receptor 1 (FGFR1), pAKT, pCREB1 (CAMP Responsive Element Binding Protein 1), human lymphocyte antigen A (HLA-A), HLA-B, or HLA-C. In some instances, the population of the MPSCs further express one or more proteins of Killer Cell Immunoglobulin Like Receptor 4 (KIR2DL4), FMS-like tyrosine kinase 3 ligand (Flt3L), NKp46, T cell receptor (TCR), Immunoglobulin-like transcript 4 (ILT-4), CD49f, CD3, CD4, CD8, CD10, CD11b, CD14, CD16, CD19, CD34, CD38, CD44, CD56, CD90/Thy-1, CD105, CD141, CD146, CD166, or CD107a. In some instances, the population of the MPSCs further express one or more proteins of interleukin 6 (IL-6), IL-8, monocyte chemoattractant protein-1 (MCP-1), CLXL2, Platelet-Derived Growth Factor AA (PDGF-AA), Vascular endothelial growth factor (VEGF), plasminogen activator inhibitor 1 (PAI-1), or IL-10. In some instances, at least some of the MPSCs do not express one or more proteins of Ki-67, heat shock protein 70 (HSP70), p53, or Syncytin. In some instances, the population of the MPSCs (*e.g.*, at least: 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%) express one or more proteins of CD44, CD90, CD105, CD146, CD166, HLA-A, HLA-B, or HLA-C. In some instances, at least some of the MPSCs do not express one or more proteins of CD19, CD45, or HLA-DR. In some instances, more than: 96%, 97%, 98% or 99% of the MPSCs do not express one or more proteins of CD19, CD45, or HLA-

DR. In some instances, the population of the MPSCs further express one or more proteins of CD16 or CD56 or a combination thereof. In some instances, at least some of the MPSCs do not express CD3. In some instances, more than: 96%, 97%, 98% or 99% of the MPSCs do not express CD3. In some instances, at least 65% or at least 70% of the population of the MPSCs express the HLA-G. In some instances, the HLA-G comprises HLA-G1, HLA-G2, HLA-G3, HLA-G4, HLA-G5, HLA-G6, or HLA-G7, or any combination thereof. In some instances, the HLA-G comprises HLA-G2, HLA-G4, HLA-G6, or HLA-G7, or any combination thereof. In some instances, the HLA-G comprises HLA-G6, or HLA-G7, or a combination thereof. In some instances, less than 15% (*e.g.*, less than 10%) of the population of the MPSCs express HLA-G1.

**[0008]** In some instances, at least 10% of the population of MPSCs disclosed herein are monoclonal. In some instances, about 13% to about 15% of the population of MPSCs are monoclonal. In some instances, at least about  $1 \times 10^6$  MPSCs are present in the population. In some instances, the MPSCs have a stable karyotype as measured by an array-based whole-genome assay. In some instances, the MPSCs experience no chromosomal aberration from population doublings, as measured by an array-based whole-genome assay. In some instances, wherein the MPSCs experience no substantial chromosomal aberration from freezing and thawing, as measured by an array-based whole-genome assay.

**[0009]** In some cases, the present disclosure provides a method of growing the population of MPSCs disclosed herein, comprising seeding a subculture of the MPSCs at a density from about 1,000 to about 5,000 cells/cm<sup>2</sup> in a culture medium, and culturing the cells.

**[0010]** In some aspects, disclosed herein is a method of growing a population of mortal pluripotent stem cells (MPSCs), comprising seeding a subculture of the MPSCs at a density from about 1,000 to about 5,000 cells/cm<sup>2</sup> in a culture medium, and culturing the cells, wherein the population of MPSCs express HLA-G and insulin. In some instances, the culture medium is free from an animal component. In some instances, the culture medium is free from serum for example fetal bovine serum. In some instances, the cells are cultured for 3 days. In some instances, the cells are cultured 4 days. In some instances, the MPSCs are seeded at a density of from about 2,000 to about 4,000 cells/cm<sup>2</sup>.

**[0011]** In some aspects, disclosed herein is a method of producing cells, comprising contacting a population of MPSCs disclosed herein with one or more inducing agents. The produced cells can comprise ectodermal cells. The produced cells can comprise mesodermal cells. The produced cells can comprise endodermal cells. The produced cells can comprise pancreatic cells or pancreatic progenitor cells and optionally the inducing agent comprises bFGF (basic fibroblast

growth factor) which may further comprise 2-mercaptoethanol and nicotinamide. In one embodiment, the PPCs comprise  $\beta$ -HCG, CDX2, HLA-G, or any combination thereof. In some instances, the PPCs comprise  $\beta$ -HCG and CDX2;  $\beta$ -HCG and HLA-G; CDX2 and HLA-G; or HCG, CDX2, and HLA-G. Optionally, in some instances, the PPCs further comprise PDX1, FOXA2, SOX9, or any combination thereof. The produced cells can comprise neural cells or neural progenitor cells and optionally the inducing agent comprises retinoic acid. In one embodiment, the NCS cells comprise retinoic acid receptor beta (RAR- $\beta$ ), CDX2, HLA-G, or any combination thereof. In some instances, the NCS cells comprise RAR- $\beta$  and CDX2; RAR- $\beta$  and HLA-G; CDX2 and HLA-G; or RAR- $\beta$ , CDX2, and HLA-G. Optionally, in some instances, the PPCs further comprise N-CAD, neuroepithelial stem cell protein (NESTIN), SRY (sex determining region Y)-box 2 (SOX2), Paired Box 6 (PAX6), or any combination thereof. The produced cells can comprise hepatic cells or hepatic progenitor cells and, optionally, the inducing agent comprises a fibroblast growth factor (FGF) such as FGF2, a steroid such as dexamethasone, and a cytokine such as oncostatin M, which may further comprise a bone morphogenetic protein (BMP), for example, BMP4, and/or a hepatic growth factor. In some instances, the FGF binds to FGFR1 and is FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF8, FGF10, FGF17, FGF19, FGF20, FGF21, FGF22, or FGF23. In some instances, the steroid is a glucocorticoid steroid, *e.g.*, dexamethasone, betamethasone, budesonide, cortisone, hydrocortisone, methylprednisolone, prednisolone, prednisone, or triamcinolone. In some instances, the cytokine is an interleukin 6 (IL-6) group cytokine, *e.g.*, oncostatin M for example a human oncostatin M, IL-6, interleukin-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), and cardiotrophin-like cytokine (CLC). The produced cells can comprise natural killer cells and the inducing agent comprises an FGF, *e.g.*, FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF8, FGF10, FGF17, FGF19, FGF20, FGF21, FGF22, or FGF23. In one embodiment, the natural killer cells are CD16<sup>+</sup>, CD56<sup>+</sup>, and CD3<sup>-</sup>. In some instances, the natural killer cells are further HLA-G<sup>+</sup> and CDX2<sup>+</sup>.

**[0012]** The produced cells can comprise adipocytes, chondrocytes, osteocytes, or any combination thereof. In one embodiment, the produced cells comprise adipocytes and chondrocytes. In another embodiment, the produced cells comprise adipocytes and osteocytes. In another embodiment, the produced cells comprise chondrocytes and osteocytes. In another embodiment, the produced cells comprise adipocytes, chondrocytes, and osteocytes.

**[0013]** In another embodiment, the produced cells comprise adipocytes. An adipocyte can comprise leptin, Homeobox C8 (HOXC8), Homeobox C9 (HOXC9), uncoupling protein 1

(Ucp1), Cell Death Inducing DFFA Like Effector A (CIDEA), PR domain containing 16 (PRDM16), Zic Family Member 1 (Zic1), LIM Homeobox 8 (Lhx8), Eva1, Epithelial Stromal Interaction 1 (Epsti1), Cd137, transmembrane protein 26 (Tmem26), T-Box Transcription Factor 1 (Tbx1), Cbp/P300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 1 (Cited1), Short Stature Homeobox 2 (Shox2), amino acid transporter ASC-1, amino acid transporter PAT2, purinergic receptor P2RX5, Adipose triglyceride lipase (ATGL), Caveolin 1 (CAV1), fatty acid binding protein 4 (FABP4), cytochrome c oxidase subunit 4 (COX4), Lamin B1 (LMNB1), or a combination thereof. In one instance, the adipocytes comprise white adipocytes, wherein the white adipocytes comprise leptin, HOXC8, HOXC9, or a combination thereof. In another instance, the adipocytes comprise brown adipocytes, wherein the brown adipocytes comprise Ucp1, CIDEA, PRDM16, Zic1, Lhx8, Eva1, Epsti1, or a combination thereof. In another instance, the adipocytes comprise beige adipocytes, wherein the beige adipocytes comprise Cd137, Tmem26, Tbx1, Cited1, Shox2, or a combination thereof. In another instance, the adipocytes comprise beige fat cell precursors, wherein the beige fat cell precursors comprise CD137, TMEM26, or a combination thereof.

**[0014]** In another embodiment, the produced cells comprise chondrocytes. A chondrocyte can comprise Annexin A6, CD44, CD151, ITM2A, Family with sequence similarity member 20-B (FAM20B), Forkhead Box C1 (FoxC1), FoxC2, SOX5, SOX6, SOX9, Aggrecan, Cathepsin B, Chondroadherin Like (CHADL), Chondroadherin, Collagen II, Collagen IV, Cartilage acidic protein 1 (CRTAC1), Dermatan sulfate proteoglycan 3 (DSPG3), Integrin Binding Sialoprotein (IBSP)/Sialoprotein II, Matrilin-1, Matrilin-3, Matrilin-4, MIA, Otoraplin/OTOR, URB, or a combination thereof.

**[0015]** In another embodiment, the produced cells comprise osteocytes. An osteocyte can comprise a pre-osteoblast, an osteoblast, embedding osteoblast, osteoid osteocyte, mineralizing osteocyte, or a mature osteocyte. An osteocyte can comprise RUNX Family Transcription Factor 2 (RUNX2), Osteocalcin (OCN), E11, Dentin Matrix Acidic Phosphoprotein 1 (DMP1), Phosphate Regulating Endopeptidase Homolog X-Linked (PHE)X, Matrix Extracellular Phosphoglycoprotein (MEPE), sclerostin, Capping Actin Protein, Gelsolin Like (CapG), ORP150, or a combination thereof. In one instance, the osteocyte comprises the pre-osteoblast, and the pre-osteoblast comprises RUNX2. In another instance, the osteocyte comprises the pre-osteoblast, and the pre-osteoblast comprises RUNX2. In another instance, the osteocyte comprises the osteoblast, and the osteoblast comprises RUNX2 and OCN. In another instance, the osteocyte comprises the embedding osteoblast, and the embedding osteoblast comprises

OCN, E11, DMP1, PHEX, and CapG. In another instance, the osteocyte comprises the osteoid osteocyte or the mineralizing osteocyte, and the osteoid osteocyte or the mineralizing osteocyte comprises OCN, E11, DMP1, PHEX, MEPE, and CapG. In another instance, the osteocyte comprises the mature osteocyte, and wherein the mature osteocyte comprises DMP1, PHEX, MPEP, Sclerostin, CapG, and ORP150.

**[0016]** In another aspect, disclosed herein is a population of mortal pluripotent stem cells (MPSCs), wherein the population of MPSCs express HLA-G, and wherein the population of MPSCs comprise a phenotype that comprises one or more of: negative for Indoleamine 2-3 deoxygenase (IDO) secretion, negative for kynurenine secretion, and positive for interleukin 2 (IL-2) secretion. In one instance, the population of MPSCs comprise a phenotype of negative for Indoleamine 2-3 deoxygenase (IDO) secretion, negative for kynurenine secretion, and positive for interleukin 2 (IL-2) secretion.

**[0017]** In any of such aspects, embodiments, and/or instances, the inventors have demonstrated stem cells are immune-privileged, chromosomally stable (not tumorigenic), pathogen free, and pluripotent. The inventors have also demonstrated efficient differentiation of its stem cells with remarkable doubling times and growth characteristics to programmed natural killer (NK), cartilage, bone, fat, neuron, pancreas, liver, and secretome cells.

#### BRIEF DESCRIPTION OF THE DRAWINGS

**[0018]** Various aspects of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

**[0019]** **Figure 1** is a line chart showing 3-day growth curves of MPSCs measured by population doublings in a time frame of 90 days.

**[0020]** **Figures 2A-2D** show a whole genome view of a KARYOSTAT™ analysis of four different MPSCs samples at different population doublings. **FIG. 2A** is a MPSC sample from 16.5 population doublings. **FIG. 2B** is a MPSC sample from 44.5 population doublings. **FIG. 2C** is a MPSC sample from 62.6 population doublings. **FIG. 2D** is a MPSC sample from 71.5 population doublings.

**[0021]** **Figure 3** shows flow cytometry analysis of MPSCs stained for HLA-G isotypes.

**[0022]** **Figure 4** shows flow cytometry analysis of MPSCs stained with a 4H84 antibody for HLA-G isotypes and with mouse IgG1 for a control.

[0023] **Figures 5A-5D** show characterization of MPSCs by expressing specific molecular biomarkers. MPSCs express molecular biomarkers such as  $\beta$ -hCG, HLA-G, HSP90, and CDX2 (**FIG. 5A**), but some are negative such as ki67, Syncytin, HSP70, p53 (**FIG. 5B**). (**FIG. 5C**) MPSCs express HLA-A, B, C (left panel) and surface and soluble HLA-G detected by 4H84 antibody (right panel) compared to isotype control and unstained cells by FACS analysis. (**FIG. 5D**) A representative FACS analysis of HLA-G isoforms in MPSCs at the cell surface compared to at the cell surface and intracellular.

[0024] **Figures 6A-6G** show expressions of molecular biomarkers of immune cells in MPSCs. By imaging or FACS analysis, MPSCs express various molecular biomarkers of NK cells (**FIG. 6A**), T cells (**FIG. 6B**), dendritic cells (**FIG. 6C** and **FIG. 6D**), macrophages (**FIG. 6E**), and stem cell progenitors (**FIG. 6F** and **FIG. 6G**). The specific biomarkers are indicated on the images or plots.

[0025] **Figure 7** provides illustrative growth curves for MPSC1 ( $\blacktriangle$ ; top line), MPSC2 ( $\blacksquare$ ), MPSC3 ( $\blacktriangledown$ ), and MPSC4 ( $\bullet$ ) over 33 passages.

[0026] **Figure 8A** illustrates the standard curve of the IDO secretion assay.

[0027] **Figure 8B** illustrates the results of IDO secretion of the three cell lines at the various concentrations of IFN- $\gamma$  stimulation compared to control. This data is “negative” indicating that there is no effect on IDO secretion compared to control.

[0028] **Figure 9A** illustrates the standard curve of the Kynurenine secretion assay.

[0029] **Figure 9B** illustrates the results of the effect of IFN- $\gamma$  stimulation on Kynurenine secretion at the three different concentrations at 24, 48, and 72 hours compared to control and media alone. This data is “negative” indicating that there is no effect on Kynurenine secretion compared to control.

[0030] **Figure 10A** illustrates the standard curve of the IL-2 secretion assay.

[0031] **Figure 10B** illustrates the results of the effect of IFN- $\gamma$  stimulation on IL-2 secretion at the three different concentrations at 24, 48, and 72 hours compared to control and media alone. This data is “positive” indicating that the cells increase IL-2 secretion compared to control.

[0032] **FIG. 10C** illustrates the effect of MPSCs seeding density of about 3000 cells/cm<sup>2</sup> at 24 hours of coculture compared to controls. Dose dependent increases were observed.

[0033] **FIG. 10D** illustrates the effect of MPSCs seeding density of about 2000 cells/cm<sup>2</sup> at 24 hours of coculture compared to controls. Dose dependent increases were observed.

[0034] **FIG. 10E** illustrates the effect of MPSCs seeding density of about 3000 cells/cm<sup>2</sup> at 48 hours of coculture compared to controls. Dose dependent increases were observed.

[0035] **FIG. 10F** illustrates the effect of MPSCs seeding density of about 2000 cells/cm<sup>2</sup> at 48 hours of coculture compared to controls. Dose dependent increases were observed. MPSCs increased, rather than decreased, IL-2 secretion by activated Jurkat cells.

[0036] **Figure 11A** is a graph illustrating cell numbers at 72 hours. In each bar, dead cells are in the top, and live cells are below.

[0037] **Figure 11B** is a graph illustrating population doublings in each type of media.

[0038] **Figure 12A** is a graph demonstrating cell counts on different days of culture.  $\Delta_{D2-D6}$  = 44,000,000 cells.

[0039] **Figure 12B** is a graph demonstrating % live cells during culture.

[0040] **Figure 12C** is a graph demonstrating population doublings comparing adherence vs. suspension cultures.  $\Delta_{D2-D6}$  = 4.9 PD. While adherent cultures initially doubled faster than suspended cultures, over time, suspended cultures achieved a higher rate of population doubling.

#### DETAILED DESCRIPTION

[0041] Disclosed herein are novel unique mortal pluripotent stem cells (MPSCs) produced *in vitro*, compositions thereof, and uses thereof in generating differentiated cells of various phenotypes (*e.g.*, pancreatic, neural, hepatic, immunoregulatory, or natural killer cell phenotype) or treating disorders (*e.g.*, diabetes, neural loss or degeneration, liver diseases, cancers, inflammations, viral infections, or autoimmune diseases) or improving conditions (*e.g.*, skin conditions). The MPSCs are distinct from previous trophoblast stem cells and have advantages including but not limited to: fast and scalable population doublings; demonstrated pathogen-free profile; being highly immune privileged and suitable for transplantation; having exceptional chromosomal stability, for example possessing stable karyotype at least to 71 population doublings; and producing robust secretome rich with cytokine, chemokines, and exosomes. The MPSCs are distinct from embryonic stem cells and are ethically sourced and cultured. Although the MPSCs are mortal (*e.g.*, having definite proliferation capacities), they are capable of reaching population doubling much faster than embryonic stem cells and iPS cells. Unlike the cells from placenta, umbilical cord, or bone marrow, the MPSCs are pluripotent and capable of differentiating or maturing into three primary group of cells that form a human being: ectoderm (giving rising to the skin, neurons, and nervous system), endoderm (forming the gastrointestinal and respiratory tracts, endocrine glands, liver or hepatocyte-like cells, and pancreas or pancreatic cells), and mesoderm (forming bone (osteocytes), adipose, cartilage (chondrocytes), most of the circulatory system, muscles, connective tissue, immune cells, and more). Furthermore, the

MPSCs are non-tumorigenic, *e.g.*, not inducing tumor or teratoma, as demonstrated in the studies of immune competent rats.

**[0042]** The details of one or more inventive embodiments are set forth in the accompanying drawings, the claims, and the description herein. Other features, objects, and advantages of the inventive embodiments disclosed and contemplated herein can be combined with any other embodiment unless explicitly excluded.

**[0043]** Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the claimed subject matter belongs. It is to be understood that the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of any subject matter claimed. In this application, the use of the singular includes the plural unless specifically stated otherwise. It must be noted that, as used in the specification and the appended claims, the singular forms “a,” “an” and “the” include plural referents unless the context clearly dictates otherwise. In this application, the use of “or” means “and/or” unless stated otherwise. Furthermore, use of the term “including” as well as other forms, such as “include”, “includes,” and “included,” is not limiting.

**[0044]** As used herein, ranges and amounts can be expressed as “about” a particular value or range, *e.g.*,  $\pm 15\%$  of a referenced numeral value. About also includes the exact amount, for example “about 5  $\mu\text{L}$ ” means “about 5  $\mu\text{L}$ ” and also “5  $\mu\text{L}$ .” Generally, the term “about” includes an amount that would be expected to be within experimental error.

**[0045]** The terms “treating,” “treatment,” and the like are used herein to mean obtaining a desired pharmacologic and/or physiologic effect. In some instances, an individual (*e.g.*, an individual suspected to be suffering from and/or genetically pre-disposed to a liver-associated disease or disorder is treated prophylactically with a preparation of cells described herein and such prophylactic treatment completely or partially prevents a liver-associated disease or disorder or sign or symptom thereof. In some instances, an individual is treated therapeutically (*e.g.*, when an individual is suffering from a liver-associated disease or disorder), such therapeutic treatment causes a partial or complete cure for the disease or disorder and/or reverses an adverse effect attributable to the disease or disorder and/or stabilizes the disease or disorder and/or delays progression of the disease or disorder and/or causes regression of the disease or disorder.

**[0046]** Administration (*e.g.*, transplantation) of cells disclosed herein to an area in need of treatment is achieved by, for example and not by way of limitation, local infusion during

surgery, by injection, by means of a catheter, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers.

**[0047]** “Transplanting” a composition into a mammal refers to introducing the composition into the body of the mammal by any method established in the art. The composition being introduced is the “transplant”, and the mammal is the “recipient”. The transplant and the recipient can be syngeneic, allogeneic or xenogeneic. Further, the transplantation can be an autologous transplantation.

**[0048]** The term “isolated,” when used in relation to a cell or a population of cells, refers to the state of the cell or population of cells being separate from and not present in a host organism, from which the cell or the population of cells may be derived. In some instances, an isolated cell is in contact with other cells that are isolated or derived from the same host organism. In some instances, an isolated cell is purified and separate from any other cells. In some instances, an isolated cell is derived *in vitro* from a stem cell.

**[0049]** An “effective amount” is an amount of a therapeutic agent sufficient to achieve the intended purpose. An effective amount of a composition to treat or ameliorate a disorder is an amount of the composition sufficient to reduce or remove the symptoms of the disorder.

**[0050]** The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

### **Cells and Compositions**

**[0051]** In some of many aspects, disclosed herein is a population of mortal pluripotent stem cells (MPSCs), wherein the population of MPSCs express HLA-G and insulin, and wherein the population of MPSCs are capable of reaching up to at least 89 population doublings within 90 days from a start of culturing the MPSCs. In some instances, the population of MPSCs are capable of reaching up to at least 89-100 population doublings within 90 days from a start of culturing the MPSCs. In some instances, the population of MPSCs are capable of reaching from about 25 to about 30 population doublings within 12 days, from about 50 to about 55 population doublings within about 30 days, and/or from about 75 to about 80 population doublings within about 63 days, from a start of culturing the MPSCs. In some instances, the population of MPSCs are capable of doubling in from about 22 to about 27 hours, for example about 25 hours. In some aspects, disclosed herein is a population of mortal pluripotent stem cells (MPSCs), wherein the population of MPSCs express HLA-G and insulin, and wherein the population of MPSCs are free from a pathogen. In some instances, the MPSC lacks expression of p53, Syncytin, Ki67,

heat shock protein 70 (HSP70), or any combination thereof. In some instances, the MPSC is a human cell. In some instances, the MPSC is originated from or derived from a rodent, rabbit, cow, sheep, pig, dog, cat, monkey, or ape.

**[0052]** In another aspect, disclosed herein is a population of mortal pluripotent stem cells (MPSCs), wherein the population of MPSCs express HLA-G, and wherein the population of MPSCs comprise a phenotype that comprises one or more of: negative for Indoleamine 2-3 deoxygenase (IDO) secretion, negative for kynurenine secretion, and positive for interleukin 2 (IL-2) secretion. In one instance, the population of MPSCs comprise a phenotype of negative for Indoleamine 2-3 deoxygenase (IDO) secretion, negative for kynurenine secretion, and positive for interleukin 2 (IL-2) secretion. This phenotype is opposite of what one of skill in the art would expect for a MPSC. While the cells look like a mesenchymal stem cell by surface phenotype markers, they behave in functionally different ways.

**[0053]** In some cases, the MPSCs disclosed herein are free from a pathogen. In some instances, the MPSCs are free from a bacterium. In some instances, the MPSCs are free from a virus, for example a cytomegalovirus. In some instances, the MPSCs are free from a pathogen selected from the group consisting of EBV (Epstein-Barr virus), HAdV (human adenovirus), HCMV (human cytomegalovirus), a Hepatitis virus (*e.g.*, Hepatitis A, Hepatitis B, Hepatitis C), a Herpes virus (*e.g.*, HHV 6 (human herpes virus 6), HHV 8 (human herpes virus 8), *etc.*), a human immunodeficiency virus (*e.g.*, HIV1 (human immunodeficiency virus 1), HIV2 (human immunodeficiency virus 2)), a human papillomavirus (HPV; *e.g.*, HPV16, HPV18, *etc.*), a herpes simplex virus (*e.g.*, HSV 1 (herpes simplex 1), HSV 2 (herpes simplex 2)), a human T-lymphotropic virus (*e.g.*, HTLV 1 (human T-lymphotropic virus 1), HTLV 2 (human T-lymphotropic virus 2)), a varicella virus (VZV), *Corynebacterium bovis*, *Corynebacterium sp.* (HAC2), a Hantavirus (*e.g.*, Hantaan, Seoul, or Sin Nombre), LCMV (lymphocytic choriomeningitis virus), *Mycoplasma sp.*, *Treponema pallidum*, a Cytomegalovirus (CMV), and any combination thereof.

**[0054]** In some instances, the MPSCs disclosed herein, or a population comprising the MPSCs, further express one or more proteins of b-HCG, HSP90, CDX2, FGFR1, pAKT, pCREB1, HLA-A, HLA-B, or HLA-C. In some instances, the population of the MPSCs further express one or more proteins of KIR2DL4, Flt3L, NKp46, TCR, ILT-4, CD49f, CD3, CD4, CD8, CD10, CD11b, CD14, CD16, CD19, CD34, CD38, CD44, CD56, CD90/Thy-1, CD105, CD141, CD146, CD166, or CD107a. In some instances, the population of the MPSCs further express one or more proteins of IL-6, IL-8, MCP-1, CLXL2, PDGF-AA, VEGF, PAI-1, or IL-10. In

some instances, at least some of the MPSCs do not express one or more proteins of Ki-67, HSP70, p53, or Syncytin. In some instances, the population of the MPSCs (*e.g.*, at least: 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99%) express one or more proteins of CD44, CD90, CD105, CD146, CD166, HLA-A, HLA-B, or HLA-C. In some instances, at least some of the MPSCs do not express one or more proteins of CD19, CD45, or HLA-DR. In some instances, more than: 96%, 97%, 98% or 99% of the MPSCs do not express one or more proteins of CD19, CD45, or HLA-DR. In some instances, the population of the MPSCs further express one or more proteins of CD16 or CD56 or a combination thereof. In some instances, at least some of the MPSCs do not express CD3. In some instances, more than: 96%, 97%, 98% or 99% of the MPSCs do not express CD3. In some instances, at least 65% or at least 70% of the population of the MPSCs express the HLA-G. In some instances, the HLA-G comprises HLA-G1, HLA-G2, HLA-G3, HLA-G4, HLA-G5, HLA-G6, or HLA-G7, or any combination thereof. In some instances, the HLA-G comprises HLA-G2, HLA-G4, HLA-G6, or HLA-G7, or any combination thereof. In some instances, the HLA-G comprises HLA-G6, or HLA-G7, or a combination thereof. In some instances, less than 15% (*e.g.*, less than 10%) of the population of the MPSCs express HLA-G1.

**[0055]** In some instances, at least 10% of the population of MPSCs disclosed herein are monoclonal. In some instances, about 13% to about 15% of the population of MPSCs are monoclonal. In some instances, at least about  $1 \times 10^6$  MPSCs are present in the population. In some instances, the MPSCs have a stable karyotype as measured by an array-based whole-genome assay. In some instances, the MPSCs experience no chromosomal aberration from population doublings, as measured by an array-based whole-genome assay. In some instances, wherein the MPSCs experience no substantial chromosomal aberration from freezing and thawing, as measured by an array-based whole-genome assay.

**[0056]** In some cases, the cells provided herein, *e.g.*, MPSCs, are genetically modified. In some instances, the cell is genetically modified to express an exogenous gene, *e.g.*, transgene. The term “transgene” and its grammatical equivalents as used herein can refer to a gene or genetic material that is transferred into an organism. For example, a transgene can be a stretch or segment of DNA containing a gene that is introduced into an organism. When a transgene is transferred into an organism, the organism is then referred to as a transgenic organism. A transgene can retain its ability to produce RNA or polypeptides (*e.g.*, proteins) in a transgenic organism. A transgene can be composed of different nucleic acids, for example RNA or DNA. A transgene may encode for an engineered T cell receptor, for example a TCR transgene. A

transgene may comprise a TCR sequence. A transgene can comprise an oncogene. A transgene can comprise an immune oncogene. A transgene can comprise recombination arms. A transgene can comprise engineered sites. In some instances, a transgene is an oncogene. In some instances, a transgene is an immune oncogene. In some instances, a transgene is a tumor suppressor gene. In some instances, a transgene encodes a protein that directly or indirectly promotes proteolysis. In some instances, a transgene is an oncolytic gene. In some instances, a transgene can aid a lymphocyte in targeting a tumor cell. In some instances, a transgene is a T cell enhancer gene. In some instances, a transgene is an oncolytic virus gene. In some instances, a transgene inhibits tumor cell growth. In some instances, a transgene is an anti-cancer receptor. In some instances, a transgene is an anti-angiogenic factor. In some instances, a transgene is a cytotoxic gene. Exemplary transgenes include, but are not limited to, CD28, inducible co-stimulator (ICOS), CD27, 4-1BB (CD137), ICOS-L, CD70, 4-1BBL, Signal 3, a cytokine such as IL-2, IL-7, IL-12, IL-15, IL-21, ICAM-1 (CD54), LFA-3 (CD58), HLA class I genes, B7, CD80, CD83, CD86, CD32, CD64, 4-1BBL, CD3, CD1d, CD2, membrane-bound IL-15, membrane-bound IL-17, membrane-bound IL-21, membrane-bound IL-2, truncated CD19, VEGF, Caspase, a chemokine, or one or more genes encoding an antibody (*e.g.*, a monoclonal antibody) to any of the above, or any combination thereof. In some instances, a transgene encodes a protein involved in cell or tissue repair (*e.g.*, proteins associated with DNA repair, the immune response (*e.g.*, interferons and interleukins), and structural proteins). In some instances, a transgene encodes a growth factor receptor. In some instances, a MPSC as described herein comprises a transgene coding for a TCR, a B cell receptor (BCR), a chimeric antigen receptor (CAR), or any combination thereof. In some instances, a MPSC as described herein comprises a transgene coding for an oncogene receptor.

**[0057]** In some cases, a composition comprising cells disclosed herein is formulated as a pharmaceutical composition for intravenous administration to a mammal, including a human. In some instances, compositions for intravenous administration are solutions in sterile tonic aqueous buffer. Where necessary, the composition also includes a local anesthetic to ameliorate any pain at the site of the injection. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients are mixed prior to administration.

**[0058]** In one aspect, disclosed herein is a composition (*e.g.*, pharmaceutical composition) comprising a cell disclosed herein. In some instances, the compositions further comprise a

pharmaceutically acceptable carrier or excipient. Such a carrier includes, but is not limited to, saline, buffered saline, dextrose, water, and combinations thereof. In other examples, a colloidal dispersion system is used. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems, including oil-in-water emulsions, micelles, mixed micelles, and liposomes.

### Methods of Use

**[0059]** In some aspects, disclosed herein is a method of producing cells, comprising contacting the population of MPSCs disclosed herein with one or more inducing agents. In some instances, the produced cells are ectodermal cells. In some instances, the produced cells are mesodermal cells. In some instances, the produced cells are endodermal cells. In some instances, the produced cells are pancreatic cells or pancreatic progenitor cells and, optionally, the inducing agent comprises bFGF (basic fibroblast growth factor), and in some instances, may further comprise 2-mercaptoethanol and nicotinamide. In one embodiment, the PPCs comprise  $\beta$ -HCG, CDX2, HLA-G, or any combination thereof. In some instances, the PPCs comprise  $\beta$ -HCG and CDX2;  $\beta$ -HCG and HLA-G; CDX2 and HLA-G; or HCG, CDX2, and HLA-G. Optionally, in some instances, the PPCs further comprise PDX1, FOXA2, SOX9, or any combination thereof. In some instances, the produced cells are neural cells or neural progenitor cells and, optionally, the inducing agent comprises retinoic acid. In one embodiment, the NCS cells comprise RAR- $\beta$ , CDX2, HLA-G, or any combination thereof. In some instances, the NCS cells comprise RAR- $\beta$  and CDX2; RAR- $\beta$  and HLA-G; CDX2 and HLA-G; or RAR- $\beta$ , CDX2 and HLA-G. Optionally, in some instances, the PPCs further comprise N-CAD, NESTIN, SOX2, PAX6, or any combination thereof. In some instances, the produced cells are hepatic cells or hepatic progenitor cells and, optionally, the inducing agent comprises a fibroblast growth factor (FGF) such as FGF2, a steroid such as dexamethasone, and a cytokine such as oncostatin M, and in some instances, may further comprise a bone morphogenetic protein (BMP) for example BMP4, and/or a hepatic growth factor. In some instances, the FGF binds to FGFR1 and is FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF8, FGF10, FGF17, FGF19, FGF20, FGF21, FGF22, or FGF23. In some instances, the steroid is a glucocorticoid steroid, *e.g.*, dexamethasone, betamethasone, budesonide, cortisone, hydrocortisone, methylprednisolone, prednisolone, prednisone, or triamcinolone. In some instances, the cytokine is an interleukin 6 group cytokine, *e.g.*, oncostatin M for example a human oncostatin M, interleukin-6, interleukin-11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), and cardiotrophin-like cytokine (CLC). In some instances, the and in some instances, cells are

natural killer cells and the inducing agent comprises an FGF, *e.g.*, FGF1, FGF2, FGF3, FGF4, FGF5, FGF6, FGF8, FGF10, FGF17, FGF19, FGF20, FGF21, FGF22, or FGF23. In some instances, MPSCs can differentiate to neural progenitor cells in 1 day with 1-step protocol, compared to a 30-day (or 3-4 weeks) and 4-step differentiation of embryonic stem cells or iPS cells. In some instances, MPSCs can differentiate into insulin-producing pancreatic progenitor cells in 1 day with 1-step protocol, compared to 8 to 15-day and 4 to 5-step differentiation of embryonic stem cells or iPS cells. In some instances, MPSCs differentiate to hepatocyte-like cells in 6 days with a 2-step protocol, compared to 12 to 21-days and 3-step differentiation of embryonic stem cells or iPS cells.

**[0060]** In one aspect, the produced cells comprise mesenchymal stromal cells that comprise adipocytes, chondrocytes, osteocytes, or any combination thereof. In one embodiment, the produced cells comprise adipocytes and chondrocytes. In another embodiment, the produced cells comprise adipocytes and osteocytes. In another embodiment, the produced cells comprise chondrocytes and osteocytes. In another embodiment, the produced cells comprise adipocytes, chondrocytes, and osteocytes.

**[0061]** In another embodiment, the produced cells comprise adipocytes. An adipocyte can comprise leptin, HOXC8, HOXC9, Ucp1, CIDEA, PRDM16, Zic1, Lhx8, Eva1, Epsti1, Cd137, Tmem26, Tbx1, Cited1, Shox2, amino acid transporter ASC-1, amino acid transporter PAT2, purinergic receptor P2RX5, ATGL, CAV1, FABP4, COX4, LMNB1, or a combination thereof. In one instance, the adipocytes comprise white adipocytes, wherein the white adipocytes comprise leptin, HOXC8, HOXC9, or a combination thereof. In another instance, the adipocytes comprise brown adipocytes, wherein the brown adipocytes comprise Ucp1, CIDEA, PRDM16, Zic1, Lhx8, Eva1, Epsti1, or a combination thereof. In another instance, the adipocytes comprise beige adipocytes, wherein the beige adipocytes comprise Cd137, Tmem26, Tbx1, Cited1, Shox2, or a combination thereof. In another instance, the adipocytes comprise beige fat cell precursors, wherein the beige fat cell precursors comprise CD137, TMEM26, or a combination thereof.

**[0062]** In another embodiment, the produced cells comprise chondrocytes. A chondrocyte can comprise Annexin A6, CD44, CD151, ITM2A, FAM20B, FoxC1, FoxC2, SOX5, SOX6, SOX9, Aggrecan, Cathepsin B, CHADL, Chondroadherin, Collagen II, Collagen IV, CRTAC1, DSPG3, IBSP/Sialoprotein II, Matrilin-1, Matrilin-3, Matrilin-4, MIA, Otoraplin/OTOR, URB, or a combination thereof.

**[0063]** In another embodiment, the produced cells comprise osteocytes. An osteocyte can comprise a pre-osteoblast, an osteoblast, embedding osteoblast, osteoid osteocyte, mineralizing osteocyte, or a mature osteocyte. An osteocyte can comprise RUNX2, OCN, E11, DMP1, PHEX, MEPE, sclerostin, CapG, ORP150, or a combination thereof. In one instance, the osteocyte comprises the pre-osteoblast, and the pre-osteoblast comprises RUNX2. In another instance, the osteocyte comprises the pre-osteoblast, and the pre-osteoblast comprises RUNX2. In another instance, the osteocyte comprises the osteoblast, and the osteoblast comprises RUNX2 and OCN. In another instance, the osteocyte comprises the embedding osteoblast, and the embedding osteoblast comprises OCN, E11, DMP1, PHEX, and CapG. In another instance, the osteocyte comprises the osteoid osteocyte or the mineralizing osteocyte, and the osteoid osteocyte or the mineralizing osteocyte comprises OCN, E11, DMP1, PHEX, MEPE, and CapG. In another instance, the osteocyte comprises the mature osteocyte, and wherein the mature osteocyte comprises DMP1, PHEX, MPEP, Sclerostin, CapG, and ORP150.

**[0064]** In some cases, a cell disclosed herein is administered to the subject intravenously, subcutaneously, percutaneously, inhalationally, orally, intramuscularly, or intratumorally. In some instances, the subject is a mammal. In some instances, the subject is a primate. In some instances, the subject is a human.

**[0065]** In some cases, disclosed herein is a method for killing an antigen-bearing target cell, comprising administering to a subject in need thereof a cell disclosed herein. In some instances, the antigen-bearing target cell is a cancer cell. In some instances, the cancer cell is a solid tumor cell. In some instances, the cancer cell is a blood cancer cell. In some instances, the cancer cell comprises a bladder cancer cell, a bone cancer cell, a brain cancer cell, a breast cancer cell, a colorectal cancer cell, an esophageal cancer cell, a gastrointestinal cancer cell, a liver cancer cell, a lung cancer cell, a nasal cancer cell, a nasopharyngeal cancer cell, an oral cancer cell, an oropharyngeal cancer cell, an ovarian cancer cell, a prostate cancer cell, a stomach cancer cell, a skin cancer cell, a thyroid cancer cell, or any combination thereof. In some instances, the cancer cell is from a cancer that comprises a hematopoietic malignancy, a head and neck squamous cell carcinoma, a leukemia, lymphoma, myeloma, sarcoma, melanoma, a bladder cancer, a bone cancer, a brain cancer, a breast cancer, a cervical cancer (e.g., a carcinoma of cervix), a colorectal cancer, an esophageal cancer, a gastrointestinal cancer, a liver cancer, a lung cancer, a nasal cancer, a nasopharyngeal cancer, an oral cancer, an oropharyngeal cancer, an ovarian cancer, a prostate cancer, a stomach cancer, a skin cancer, a thyroid cancer, or any combination thereof. The cancer comprises a primary cancer. Alternatively, the cancer comprises a metastatic

cancer. In some instances, the antigen-bearing target cell is a pathogen. In some instances, the pathogen comprises a virus, a bacterium, a protozoa, a prion, a fungus, or any combination thereof. In some instances, the method kills at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or 100% of a population of antigen-bearing target cells. In some instances, the method kills about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, about 99%, or 100% of a population of antigen-bearing target cells.

**[0066]** In some cases, disclosed herein is a method for downregulating an inflammatory pathway, comprising administering to a subject in need thereof a cell disclosed herein. In some instances, the method treats a disease or condition that comprises a transplant rejection, an infection, endotoxic shock associated with infection, arthritis, rheumatoid arthritis, psoriatic arthritis, systemic onset juvenile idiopathic arthritis (JIA), inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE), asthma, pelvic inflammatory disease, Alzheimer's disease, Crohn's disease, ulcerative colitis, irritable bowel syndrome, multiple sclerosis, ankylosing spondylitis, dermatomyositis, uveitis, Peyronie's disease, coeliac disease, gallbladder disease, Pilonidal disease, peritonitis, psoriasis, vasculitis, surgical adhesions, stroke, Type I diabetes, Lyme arthritis, meningoencephalitis, immune mediated inflammatory disorders of the central and peripheral nervous system, pancreatitis, trauma from surgery, graft-versus-host disease, heart disease, bone resorption, burns patients, myocardial infarction, Paget's disease, osteoporosis, sepsis, liver or lung fibrosis, periodontitis, or hypochlorhydria. In some instances, the method treats an autoimmune disease that comprises Type I diabetes, multiple sclerosis, systemic lupus erythematosus, Sjogren's syndrome, scleroderma, polymyositis, chronic active hepatitis, mixed connective tissue disease, primary biliary cirrhosis, pernicious anemia, autoimmune thyroiditis, idiopathic Addison's disease, vitiligo, gluten-sensitive enteropathy, Graves' disease, myasthenia gravis, autoimmune neutropenia, idiopathic thrombocytopenia purpura, rheumatoid arthritis, cirrhosis, pemphigus vulgaris, autoimmune infertility, Goodpasture's disease, bullous pemphigoid, discoid lupus, ulcerative colitis, dense deposit disease, inflammatory bowel disease, psoriasis, or any combination thereof. In some instances, the method treats Type 1 diabetes. In some instances, the method ameliorates transplant rejection.

**[0067]** In another aspect, disclosed herein is a method of treating a condition in a subject, comprising administering to a subject a pharmaceutical composition that comprises a cell herein, in an amount effective for the cells to engraft to the subject (*e.g.*, to the subject's liver). In some instances, the cells are administered in a pharmaceutically acceptable carrier. In some instances, the pharmaceutically acceptable carrier comprises a saline for example a phosphate buffer saline, or fetal bovine serum. In some instances, the cells are administered in a suspension containing from about  $1 \times 10^6$  to about  $100 \times 10^6$  cells per ml, from about  $1 \times 10^6$  to about  $250 \times 10^6$  cells per ml, from about  $1 \times 10^6$  to about  $500 \times 10^6$  cells per ml, or from about  $10 \times 10^6$  to about  $40 \times 10^6$  cells per ml. In some instances, the cells are administered in a volume of about: 1-5 ml, 1-10 ml, 1-50 ml, 1-100 ml, or 10-150 ml. In some instances, the subject is a human. In some instances, the administering comprises an injection, *e.g.*, intravenous injection. In some instances, the injection is administered at a hepatic vein. In some instances, the injection is administered at a hepatic artery. In some instances, the condition is a liver-associated disease or disorder, *e.g.*, acute liver disease. In some instances, the condition is a liver failure. In some instances, the liver-associated disease or disorder comprises Alagille syndrome, alpha 1 anti-trypsin deficiency, autoimmune hepatitis, benign liver tumors, biliary atresia, cirrhosis, cystic disease of the liver, fatty liver disease including alcohol-related liver disease and non-alcohol fatty liver disease (NAFLD), galactosemia, gallstones, Gilbert's syndrome, hemochromatosis, liver cysts, liver cancer, liver disease in pregnancy (optionally, acute fatty liver of pregnancy, intrahepatic cholestasis of pregnancy, preeclampsia, or HELLP syndrome (*e.g.*, hemolysis, elevated liver tests, low platelets)), neonatal hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, porphyria, Reye's syndrome, sarcoidosis, toxic hepatitis, type 1 glycogen storage disease, tyrosinemia, viral hepatitis, Wilson disease, or any combination thereof.

**[0068]** Modes of administration of cells disclosed herein include, but are not limited to, systemic intravenous injection and injection directly to the intended site of activity (*e.g.*, endoscopic retrograde injection). The preparation can be administered by any convenient route, for example, by infusion or bolus injection, and can be administered together with other biologically active agents. In some instances, the administration is systemic localized administration.

**[0069]** In some aspects, provided herein are compositions and methods for transplanting cells disclosed herein to subjects. In some instances, the subject is injected by the cells (*e.g.*, intravenously, intramuscularly, transdermally, endoscopic retrograde injection, or intraperitoneally). In some instances, the subject is not treated with an immunosuppressive agent prior to the transplanting. In some instances, the method further comprises treating the patient

with an immunosuppressive agent, *e.g.*, FK-506, cyclosporin, or an anti-glutamic acid decarboxylase 65-kilodalton isoform (GAD65) antibody.

**[0070]** In some instances, cells described herein are delivered to a targeted site (*e.g.*, a defect section of the liver) by a delivery system suitable for targeting cells to a particular tissue. For example, the cells are encapsulated in a delivery vehicle that allows for the slow release of the cell(s) at the targeted site. The delivery vehicle can be modified such that it is specifically targeted to a particular tissue. The surface of the targeted delivery system can be modified in a variety of ways. In the case of a liposomal-targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer.

**[0071]** The administration of cells described herein can be, optionally, tailored to an individual, by: (1) increasing or decreasing the amount cells injected; (2) varying the number of injections; or (3) varying the method of delivery of the cells.

#### **Detection Methods**

**[0072]** Methods for determining the expression or presence of biomarkers described *supra* are well known in the art, and can be measured, for example, by flow cytometry, immunohistochemistry, western blot, immunoprecipitation, magnetic bead selection, and quantification of cells expressing either of these cell surface markers. Biomarker RNA expression levels can be measured, for example, using RT-PCR, Qt-PCR, microarray, northern blot, or other similar technologies.

**[0073]** By “detecting expression” or detecting “expression levels” is intended for determining the expression level or presence of a biomarker protein or gene in the biological sample. Thus, “detecting expression” encompasses instances where a biomarker is determined not to be expressed, not to be detectably expressed, expressed at a low level, expressed at a normal level, or overexpressed.

**[0074]** In some instances, the expression or presence of a biomarker described herein is determined at a nucleic acid level, using, for example, immunohistochemistry techniques or nucleic acid-based techniques such as *in situ* hybridization and RT-PCR. In some instances, the expression or presence of one or more biomarkers is carried out by a means for nucleic acid amplification, a means for nucleic acid sequencing, a means utilizing a nucleic acid microarray (DNA and RNA), or a means for *in situ* hybridization using specifically labeled probes.

**[0075]** In some instances, the determining the expression or presence of a biomarker is carried out through gel electrophoresis. In some instances, the determination is carried out through

transfer to a membrane and hybridization with a specific probe. In some instances, the determining the expression or presence of a biomarker is carried out by a diagnostic imaging technique. In some instances, the determining the expression or presence of a biomarker is carried out by a detectable solid substrate. In some instances, the detectable solid substrate is paramagnetic nanoparticles functionalized with antibodies.

**[0076]** In some instances, the expression or presence of a biomarker is at an RNA (*e.g.* mRNA) level. In some instances, techniques that detect RNA (*e.g.* mRNA) level include, but are not limited to, Southern or Northern analyses, polymerase chain reaction analyses and probe arrays.

**[0077]** One method for the detection of mRNA levels involves contacting the isolated mRNA with a nucleic acid molecule (probe) that hybridize to the mRNA encoded by the gene being detected. The nucleic acid probe comprises of, for example, a full-length cDNA, or a portion thereof, such as an oligonucleotide of at least 7, 15, 30, 50, 100, 250, or 500 nucleotides in length and sufficient to specifically hybridize under stringent conditions to an mRNA or genomic DNA encoding a biomarker described herein. Hybridization of an mRNA with the probe indicates that the biomarker or other target protein of interest is being expressed.

**[0078]** In some instances, the mRNA is immobilized on a solid surface and contacted with a probe, for example by running the isolated mRNA on an agarose gel and transferring the mRNA from the gel to a membrane, such as nitrocellulose. In some instances, the probe(s) are immobilized on a solid surface and the mRNA is contacted with the probe(s), for example, in a gene chip array. A skilled artisan readily adapts known mRNA detection methods for use in detecting the level of mRNA encoding the biomarkers or other proteins of interest.

**[0079]** An alternative method for determining the level of an mRNA of interest in a sample involves the process of nucleic acid amplification, *e.g.*, by RT-PCR, ligase chain reaction, self-sustained sequence replication, transcriptional amplification system, Q-Beta Replicase, rolling circle replication or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers. In some instances, biomarker expression is assessed by quantitative fluorogenic RT-PCR (*e.g.*, the TAQMAN® System).

**[0080]** Expression levels of an RNA of interest are monitored using a membrane blot (such as used in hybridization analysis such as northern, dot, and the like), or microwells, sample tubes, gels, beads or fibers (or any solid support comprising bound nucleic acids). The detection of expression also comprises using nucleic acid probes in solution.

**[0081]** In some instances, microarrays are used to determine expression or presence of one or more biomarkers. Nucleic acid microarrays provide one method for the simultaneous measurement of the expression levels of large numbers of genes. Each array consists of a reproducible pattern of capture probes attached to a solid support. Labeled RNA or DNA is hybridized to complementary probes on the array and then detected by laser scanning. Hybridization intensities for each probe on the array are determined and converted to a quantitative value representing relative gene expression levels. High-density oligonucleotide arrays are particularly useful for determining the gene expression profile for a large number of RNAs in a sample.

**[0082]** In some instances, an array is fabricated on a surface of virtually any shape or even a multiplicity of surfaces. In some instances, an array is a planar array surface. In some instances, arrays include peptides or nucleic acids on beads, gels, polymeric surfaces, fibers such as fiber optics, glass or any other appropriate substrate. In some instances, arrays are packaged in such a manner as to allow for diagnostics or other manipulation of an all-inclusive device.

**[0083]** In some instances, the expression or presence of a biomarker described herein is determined at a protein level, using, for example, antibodies that are directed against specific biomarker proteins. These antibodies are used in various methods such as western blot, ELISA, multiplexing technologies, immunoprecipitation, or immunohistochemistry techniques. In some instances, detection of biomarkers is accomplished by ELISA. In some instances, detection of biomarkers is accomplished by electrochemiluminescence (ECL).

**[0084]** Any means for specifically identifying and quantifying a biomarker in the biological sample is contemplated. Thus, in some instances, expression level of a biomarker protein of interest in a biological sample is detected by means of a binding protein capable of interacting specifically with that biomarker protein or a biologically active variant thereof. In some instances, labeled antibodies, binding portions thereof, or other binding partners are used. The word “label” when used herein refers to a detectable compound or composition that is conjugated directly or indirectly to the antibody so as to generate a “labeled” antibody. In some instances, the label is detectable by itself (*e.g.*, radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, catalyzes chemical alteration of a substrate compound or composition that is detectable.

**[0085]** The antibodies for detection of a biomarker protein are either monoclonal or polyclonal in origin, or are synthetically or recombinantly produced. The amount of complexed protein, for example, the amount of biomarker protein associated with the binding protein, for example, an

antibody that specifically binds to the biomarker protein, is determined using standard protein detection methodologies known to those of skill in the art. A detailed review of immunological assay design, theory and protocols are found in numerous texts in the art.

**[0086]** The choice of marker used to label the antibodies will vary depending upon the application. However, the choice of the marker is readily determinable to one skilled in the art. These labeled antibodies are used in immunoassays as well as in histological applications to detect the presence of any biomarker or protein of interest. The labeled antibodies are either polyclonal or monoclonal. Further, the antibodies for use in detecting a protein of interest are labeled with a radioactive atom, an enzyme, a chromophoric or fluorescent moiety, or a colorimetric tag as described elsewhere herein. The choice of tagging label also will depend on the detection limitations desired. Enzyme assays (*e.g.*, ELISAs) typically allow detection of a colored product formed by interaction of the enzyme-tagged complex with an enzyme substrate. Radionuclides that serve as detectable labels include, for example, I-131, I-123, I-125, Y-90, Re-188, Re-186, At-211, Cu-67, Bi-212, and Pd-109. Examples of enzymes that serve as detectable labels include, but are not limited to, horseradish peroxidase, alkaline phosphatase, beta-galactosidase, and glucose-6-phosphate dehydrogenase. Chromophoric moieties include, but are not limited to, fluorescein and rhodamine. The antibodies are conjugated to these labels by methods known in the art. For example, enzymes and chromophoric molecules are conjugated to the antibodies by means of coupling agents, such as dialdehydes, carbodiimides, dimaleimides, and the like. Alternatively, conjugation occurs through a ligand-receptor pair. Examples of suitable ligand-receptor pairs include, but are not limited to, biotin-avidin or biotin-streptavidin, and antibody-antigen.

**[0087]** In some instances, expression or presence of one or more biomarkers or other proteins of interest within a biological sample is determined by radioimmunoassays or enzyme-linked immunoassays (ELISAs), competitive binding enzyme-linked immunoassays, dot blot, western blot, chromatography such as high performance liquid chromatography (HPLC), or other assays known in the art. Thus, the detection assays involve steps such as, but not limited to, immunoblotting, immunodiffusion, immunoelectrophoresis, and immunoprecipitation.

#### **Methods of Obtaining Cells**

**[0088]** In some cases, an extra-embryonic mammalian stem cell (*e.g.*, a trophoblast stem cell) can be a source cell to make mortal pluripotent stem cells (MPSCs) disclosed herein. In some instances, the mammalian stem cells are isolated from amniotic fluid, amniotic membrane,

Wharton's jelly, chorionic villi, or an ectopic pregnancy, in a manner that is not disturbing nor destructive to an embryo.

**[0089]** In some instances, the MPSC is obtained in a culture medium free from an antibiotic, for instance, penicillin, streptomycin, or any combination thereof. In some instances, the culture medium for obtaining the mammalian stem cell is free from retinoic acid. In some instances, the culture medium obtaining and/or passaging the mammalian stem cell is free from mercaptoethanol, nicotinamide, or a combination thereof. In some instances, the culture medium obtaining and/or passaging the mammalian stem cell is free from dexamethasone, recombinant human oncostatin M, BMP4, HGF, or any combination thereof. In some instances, the culture medium obtaining and/or passaging the mammalian stem cell is xeno-free, *e.g.*, free from an animal component. In some instances, the culture medium obtaining and/or passaging the mammalian stem cell is free from a human derived component and an animal-derived component, *e.g.*, a chemically defined medium. In some instances, the culture medium obtaining and/or passaging the mammalian stem cell is free from a serum. In some instances, the culture medium obtaining and/or passaging the mammalian stem cell is free from fetal bovine serum.

**[0090]** In some cases, the present disclosure provides a method of growing the population of MPSCs disclosed herein, comprising seeding a subculture of the MPSCs at a density from about 1,000 to about 5,000 cells/cm<sup>2</sup> in a culture medium, and culturing the cells.

**[0091]** In some aspects, disclosed herein is a method of growing a population of mortal pluripotent stem cells (MPSCs), comprising seeding a subculture of the MPSCs at a density from about 1,000 to about 5,000 cells/cm<sup>2</sup> in a culture medium, and culturing the cells, wherein the population of MPSCs express HLA-G and insulin. In some instances, the culture medium is free from an animal component. In some instances, the culture medium is free from serum for example fetal bovine serum. In some instances, the cells are cultured for about 3 days. In some instances, the cells are cultured for about 4 days. In some instances, the MPSCs are seeded at a density of from about 2,000 to about 4,000 cells/cm<sup>2</sup>.

**[0092]** In some instances, the mammalian stem cell can be isolated from amniocentesis biopsies or from amniotic fluid. In one instance, amniocentesis can be a procedure used to obtain a small sample of the amniotic fluid that surrounds the fetus during pregnancy. In one instance, an amniocentesis can be offered to women between the 15th and 20th weeks of pregnancy who are at increased risk for chromosome abnormalities, *e.g.*, women who are over 35 years of age at delivery, or those who have had an abnormal maternal serum (blood) screening test indicating an increased risk for a chromosomal abnormality or neural tube defect. In one instance, a needle,

*e.g.*, a long, thin, hollow needle, can be used with ultrasound guide through your abdomen, into the uterus and the amniotic sac. A predetermined amount of amniotic fluid, *e.g.* one ounce, can be drawn into a syringe.

**[0093]** In some instances, the mammalian stem cell herein can be obtained from blastomere biopsy during preimplantation genetic diagnosis (PGD), *e.g.*, in conjunction with reproductive therapies such as in vitro fertilization (IVF). In one instance, the cells herein can be produced by methods for biopsy of a blastocyst, wherein the remainder of the blastocyst is implanted and results in a pregnancy and later in a live birth, *e.g.*, the zona pellucida is removed from the blastocyst and then the blastocyst is biopsied.

**[0094]** In some instances, a mammalian stem cell herein can be obtained from prenatal chorionic villus sampling (CVS). In one instance, CVS can be a prenatal test that involves taking a sample of tissue from the placenta to test for chromosomal abnormalities and certain other genetic problems. In one instance, CVS can be performed between the 10th and 12th weeks of pregnancy. In one instance, the CVS procedure is transcervical, *e.g.*, a catheter is inserted through the cervix into the placenta to obtain the tissue sample. In one instance, the CVS procedure is transabdominal, *e.g.*, a needle is inserted through the abdomen and uterus into the placenta to obtain the tissue sample.

**[0095]** In some instances, the mammalian stem cell herein can be isolated from first trimester chorionic villous sampling (*e.g.*, 8<sup>+3</sup> to 12<sup>+0</sup> weeks gestational age) or term placenta from caesarean section deliveries. The chorionic tissue can be separated from the amnion, minced, and/or enzymatically digested (*e.g.*, with about 3 ml TRYPLE® Select Enzyme, *e.g.*, for about 15 min). Cells are subsequently centrifuged (*e.g.*, at about 150 × g +/- 10%, *e.g.*, for about 5 min), counted, and/or replated (*e.g.*, about 100 cells per cm<sup>2</sup>) in a medium (*e.g.*, α-MEM with STEMULATE™ Human Platelet Lysate Cell Culture Media Supplement or MESENCULT™-ACF Plus Culture Kit). In one instance, isolated cells can be plastic adherent. In one instance, the cells can be used at a passage of from about 4 to about 8.

**[0096]** In some instances, chorionic villi can be obtained from the fallopian tubes of un-ruptured pre-implantation embryos in women with ectopic pregnancy (*e.g.*, gestational age: from about 5 to about 8 weeks, from about 6 to about 8 weeks, or to about 4 to about 8 weeks post fertilization). Tiny villous tissues can be well-minced in a suitable medium (*e.g.*, serum-free α-MEM) and identified under microscopy followed by trypsinization (*e.g.*, with about 3 ml TRYPLE® Select Enzyme) for a period of time (*e.g.*, about 15 min) and by adding a medium (*e.g.*, α-MEM with STEMULATE™ Human Platelet Lysate Cell Culture Media Supplement or

MESENCULT™-ACF Plus Culture Kit) to halt the reaction. Adherent cells can be obtained and cultured in a suitable condition (*e.g.*, in conditioned  $\alpha$ -MEM with STEMULATE™ Human Platelet Lysate Cell Culture Media Supplement or MESENCULT™-ACF Plus Culture Kit at 37 °C in 5% CO<sub>2</sub>).

#### **Kits/Articles of Manufacture**

[0097] Disclosed herein are kits and articles of manufacture for use with one or more methods and compositions described herein. Such kits include a carrier, package, or container that is compartmentalized to receive one or more containers such as vials, tubes, and the like, each of the container(s) comprising one of the separate elements to be used in a method described herein. Suitable containers include, for example, bottles, vials, syringes, test tubes, *etc.* In some instances, the containers are formed from a variety of materials such as glass or plastic.

[0098] The articles of manufacture provided herein contain packaging materials. Examples of pharmaceutical packaging materials include, but are not limited to, blister packs, bottles, tubes, bags, containers, bottles, and any packaging material suitable for a selected formulation and intended mode of use.

[0099] For example, the container(s) include cells, optionally in a composition as disclosed herein. Such kits optionally include an identifying description or label or instructions relating to its use in the methods described herein.

[00100] A kit typically includes labels listing contents and/or instructions for use, and package inserts with instructions for use. A set of instructions will also typically be included.

[00101] In some instances, a label is on or associated with the container. In some instances, a label is on a container when letters, numbers or other characters forming the label are attached, molded or etched into the container itself; a label is associated with a container when it is present within a receptacle or carrier that also holds the container, *e.g.*, as a package insert. In some instances, a label is used to indicate that the contents are to be used for a specific therapeutic application. The label also indicates directions for use of the contents, such as in the methods described herein.

#### **Medicaments, Compositions, and Uses Thereof**

[00102] Disclosed herein are compositions (*e.g.*, *in vitro* compositions, pharmaceutical compositions, *etc.*) and medicaments comprising the cells produced by the methods described herein. Compositions or medicaments having a desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington, The Science and Practice of Pharmacy 20th *Ed.* Mack Publishing (2000)), may be prepared in the form of

lyophilized formulations or aqueous solutions. As used herein, “pharmaceutically acceptable carrier” or “pharmaceutical acceptable excipient” includes any material which, when combined with an active ingredient, allows the ingredient to retain biological activity and is non-reactive with the subject's immune system. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Preferred diluents for aerosol or parenteral administration are phosphate buffered saline or normal (0.9%) saline. Compositions comprising such carriers are formulated by well-known conventional methods (*see*, for example, Remington's Pharmaceutical Sciences, 18th edition, A. Gennaro, *Ed.*, Mack Publishing Co., Easton, Pa., 1990; and Remington, The Science and Practice of Pharmacy 20th *Ed.* Mack Publishing, 2000).

**[00103]** Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and may comprise buffers such as phosphate, citrate, and other organic acids; salts such as sodium chloride; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens, such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (*e.g.*, Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

**[00104]** Described herein is a use of a produced cell described herein for *in vitro* cultures or assays. For example, the cells can be for use in an immunofluorescence or fluorescent activated cell sorting (FACS) assay. In some instances, the produced cell can be a neural stem cell (NCS), a pancreatic progenitor cell (PPC), an ectodermal cell, a mesodermal cell, an endodermal cell, a hepatic cell, or a hepatic progenitor cell.

**[00105]** Described herein is a use of neural stem cells (NSCs) produced by a method described herein to test new drugs for safety and effectiveness. For example, a test agent can be contacted with a culture comprising the produced cells and the effect determined. If a test agent

is toxic to cells, proliferation of the culture may decrease and/or die. If a test agent is efficacious, proliferation of the culture may increase. In the case of neural stem cells, a test agent may induce production of motor neurons. Described herein is a use of neural stem cells (NSCs) produced by a method described herein to produce motor neurons *in vitro* or *in vivo*. Described herein is a use of neural stem cells (NSCs) produced by a method described herein in the manufacture of a medicament for the treatment of a motor neuron disease. Described herein is a use of neural stem cells (NSCs) produced by a method described herein in the manufacture of a medicament for the treatment of a spinal cord injury. Described herein is a use of neural stem cells (NSCs) produced by a method described herein to produce an artificial tissue or organ *in vitro*.

**[00106]** Described herein is a use of pancreatic progenitor cells (PPCs) produced by a method described herein to test new drugs for safety and effectiveness. For example, a test agent can be contacted with a culture comprising the produced cells and the effect determined. If a test agent is toxic to cells, proliferation of the culture may decrease and/or die. If a test agent is efficacious, proliferation of the culture may increase. In the case of PPCs, a test agent may induce production of endocrine cells and/or exocrine cells. Described herein is a use of PPCs produced by a method described herein to produce endocrine cells and/or exocrine cells *in vitro* or *in vivo*. Described herein is a use of PPCs produced by a method described herein in the manufacture of a medicament for the treatment of a disease or disorder cause by a pancreatic injury. Described herein is a use of PPCs produced by a method described herein in the manufacture of a medicament for the treatment of a pancreatic injury. Described herein is a use of PPCs produced by a method described herein to produce an artificial tissue or organ *in vitro* (*e.g.*, a pancreas).

## EXAMPLES

**[00107]** The application may be better understood by reference to the following non-limiting examples, which are provided as exemplary embodiments of the application. The following examples are presented in order to more fully illustrate embodiments and should in no way be construed, however, as limiting the broad scope of the application.

### **Example 1. MPSCs reached 89 population doublings**

**[00108]** Extra-embryonic stem cells (for example, trophoblast stem cells) came from human donors as source cells. Several non-limiting culture media were tested to culture the cells to grow into mortal pluripotent stem cells (MPSCs), as shown in Table 1 below.

**[00109] Table 1. Culture media for growing MPSCs.**

| Medium                                                    | Supplements                                                                                    | Coating                                  |
|-----------------------------------------------------------|------------------------------------------------------------------------------------------------|------------------------------------------|
| MEM Alpha + GlutaMAX<br>(Gibco 32571-036)                 | STEMULATE 10% Human Platelet Lysate Cell Culture Media Supplement<br>(Cook Regentec PL-NH-100) | -                                        |
| MEM Alpha + GlutaMAX<br>(Gibco 32571-036)                 | STEMULATE 10% Human Platelet Lysate Cell Culture Media Supplement<br>(Cook Regentec PL-NH-100) | CELLBIND® surface (Corning)              |
| MEM Alpha + GlutaMAX<br>(Gibco 32571-036)                 | STEMULATE 10% Human Platelet Lysate Cell Culture Media Supplement<br>(Cook Regentec PL-NH-100) | Laminin<br>(0.5 µg/cm <sup>2</sup> )     |
| MESENCULT™-ACF Plus<br>(StemCell Technologies, 05448)     | MESENCULT™-ACF Plus 500x Supplement, Glutamine 2mM                                             | Cell Attachment Substrate (CAS)          |
| STEMPRO® MSC SFM XenoFree<br>(Gibco, A1067501)            | STEMPRO® MSC SFM XenoFree Supplement<br>Glutamine 2 mM                                         | Fibronectin<br>(0.4 µg/cm <sup>2</sup> ) |
| RoosterNourish-MSC-XF<br>(RoosterBio, KT-016)             | ROOSTER BOOSTER® Supplement                                                                    | CELLBIND® surface (Corning)              |
| Prime XV MSC Expansion XSFM<br>(Irvine Scientific, 91149) | -                                                                                              | Fibronectin<br>(0.4 µg/cm <sup>2</sup> ) |
| Prime XV NK cell CDM<br>(Irvine Scientific, 91215)        | rhIL2-ACF 550 IU/ML                                                                            | -                                        |

**[00110]** Lowering seeding density from 10,000 to 2,000 - 4,000 cells/cm<sup>2</sup> improved the number of population doublings for MPSCs. 3-day subcultures of cells seeded at densities between 3000 – 5000 cells/cm<sup>2</sup> generate similar numbers of PD. Alternating 3-day/4-day subcultures of cells seeded at 4000/3000 cells/cm<sup>2</sup> generates similar numbers of PD as 3-day subcultures at 4000 cells/cm<sup>2</sup> for earlier passages. Culture environment can be 21% O<sub>2</sub>/ % CO<sub>2</sub>, or 2% O<sub>2</sub>/ 5% CO<sub>2</sub>/ 93% N<sub>2</sub>.

**[00111]** Some of the growth results over 90 days, i.e., 30 passages of 3-day-subcultures, are displayed in **Figure 1**, which is a line chart showing that 3-day-subculture growth curves of MPSCs measured by population doublings (PD) in a time frame of 90 days. The MPSCs reached up to 25 PD by about 12 days, up to 50 PD by about 30 days, up to 75 PD by about 63 days, up to 89 PD by about 90 days.

**[00112]** MPSCs possess an extended population doubling capacity of ~70-80 doublings with a doubling time of ~27 hours when cultured in xeno-free media. It takes about averagely 27 hours for the cells to double in population, which can be calculated with an equation  $T = t_d / \log_2[(2 - y)/(1 - y)]$ , where  $T$  is the duration of the cell cycle,  $t_d$  is the average time of duplication of cell number, and  $y$  is proportion of cells in G0 phase.

**[00113]** This extended doubling capacity make MPSCs an ideal population of cells for expansion at industrial scale, eliminating the need for repeated isolation from a donor or biological source. The large batch size potential of MPSCs would generate sufficient MPSCs from a single derivation, streamlining therapeutic development and manufacturing and accelerating the realization of stem cell based therapeutics in the clinic by reducing product variability associated with multiple banks and the costs of manufacturing and releasing comparable product from different donors.

**Example 2. MPSCs have no chromosomal aberrations**

**[00114]** **Figures 2A-2D** show a whole genome view of a KARYOSTAT™ from four different MPSC samples from different population doublings. A KARYOSTAT™ assay can allow for digital visualization of chromosome aberrations. The size of structural aberration that can be detected is about > 2 Mb (megabase) for chromosomal gains and about > 1 Mb for chromosomal losses. Genomic DNA was purified from cells and the genomic DNA was added to the GENECHIP® for the KARYOTATE™. The GENECHIP® can determine copy number variants of chromosomes. **Figures 2A-2D** show the whole genome view that displays all somatic and sex chromosomes in one frame. **Figure 2A**, is a MPSC sample from 16.5 population doublings, **Figure 2B**, is a MPSC sample from 44.5 population doublings, **Figure 2C** is a MPSC sample from 62.6 population doublings, and **Figure 2D** is a MPSC sample from 71.5 population doublings. The smooth signal plot (right y-axis) is the smoothing of the log2 ratios which depict the signal intensities of probes on the microarray. A value of 2 can represent a normal copy number state (CN = 2). A value of 3 can represent a chromosomal gain (CN = 3). A value of 1 can represent a chromosomal loss (CN = 1). The gray signal indicates the raw signal for each individual chromosome probe, while the black signal represents the normalized probe signal which is used to identify copy number and aberrations (if any). No observable chromosomal aberrations were found in **Figures 2A-D**. The MPSC cells can go through multiple population doublings without chromosomal aberrations. For example, a monoclonal population could be expanded and then frozen for future use. Once the cells are grown from the frozen stock cultures of monoclonal they can be expanded without substantial chromosomal

aberrations. The chromosome stability of MPCs provides another advantage over human embryonic stem cells and iPSCs which often show genetic abnormalities or mutations associated with immortality.

**Example 3. Characterization of MPSCs by expressing specific molecular biomarkers**

**[00115]** MPSCs express an immune-privilege marker HLA-G. Unlike adult or post-natal human mesenchymal stromal cells, MPSCs herein express the human leukocyte antigen-G (HLA-G), a major histocompatibility complex class I antigen exclusive to the placenta that binds to HLA-G receptors on leukocytes to suppress immune function via numerous mechanisms, including triggering apoptosis in activated T cells, modulating the activity of Natural Killer (NK) cells and dendritic cells, and inhibiting T-cell proliferation. Referring to **Figure 3**, this figure shows MPSCs stained with the primary antibody 4H84. For **Figure 3**, the MPSCs were harvested from culture in MESENCULT® ACF Plus Medium. Cells were resuspended in flow cytometry wash buffer (Gibco DPBS, substantially without calcium chloride or magnesium chloride, about 2% fetal bovine serum and about 0.1% sodium azide) and aliquoted from about  $0.25\text{--}0.5 \times 10^6$  cells per sample into a flow cytometry tube and the cells were centrifuged. The cells were fixed with a 4% paraformaldehyde solution for about 15 min at room temperature. In some instances, after fixation the cells were permeabilized with about 500  $\mu\text{l}$  (microliters) of cold Perm Buffer III (BD Biosciences) and then the cells were incubated on ice. The permeabilization permitted intracellular material to be stained. After the incubation, the cells were washed with flow cytometry wash buffer, centrifuged and resuspended in flow cytometry staining buffer (R&D Systems). The primary antibody staining occurred when a dilution of an HLA-G primary antibody (*e.g.* 4H84 antibody) was added to the cells and incubated at room temperature. The cells were washed several times with flow cytometry wash buffer. After the primary antibody had been bound to the cells, a secondary antibody was added. The secondary antibody procedure took place in the dark. The secondary antibody was added at a dilution of about 1:2000 into flow cytometry staining buffer. The cells were resuspended in about 100  $\mu\text{l}$  of diluted secondary antibody solution and incubated for about 30 minutes. The cells were washed several times in flow cytometry wash buffer. After the cells were washed, the cells were resuspended in flow cytometry staining buffer to a concentration of about  $0.5 \times 10^6$  cells. After the cells were resuspended, the cells were sampled by flow cytometry. The primary antibody MEM-G/11 can recognize the HLA-G1 isoform which is membrane bound. The primary antibody 4H84 antibody can recognize the alpha domain of 7 isoforms of HLA-G. **Figure 3** shows MPSCs permeabilized and stained with the primary antibody HLA-G 4H84. The staining

shows the presence of HLA-G isoforms in or on MPSCs. The staining shows about 76% of the cells in a 1:50 primary antibody dilution were positive compared to an isotype control. **Figure 4** shows the cells stained with the primary antibody 4H84 (bottom panel) and the primary isotype control antibody mouse IgG1 (top panel). The cells show limited staining with the IgG1 antibody and 99.64% of events were stained with the 4H84 antibody, which indicates the antibody is specific to MPSCs. The expression of HLA-G in MPSCs as shown in **Figure 3**, may allow the cells to have access to immune privileged sites, for example a fetus.

**[00116]** The MPSCs herein have shown human MSC phenotype and morphology by expressing the characteristic markers as measured by fluorescence activated cell sorting (FACS), *see* Table 2 below.

**[00117]** **Table 2. Expression of the markers showing mesenchymal stromal cell – like phenotype**

| PD          | NEGATIVE MARKERS |             |                | POSITIVE MARKERS |             |              |              |              |                      |
|-------------|------------------|-------------|----------------|------------------|-------------|--------------|--------------|--------------|----------------------|
|             | CD19<br>(-)      | CD45<br>(-) | HLA-<br>DR (-) | CD44<br>(+)      | CD90<br>(+) | CD105<br>(+) | CD146<br>(+) | CD166<br>(+) | HLA-<br>A,B,C<br>(+) |
| <b>19.8</b> | 99.9             | 98.6        | 99.6           | 99.7             | 99.8        | 96.6         | 92.1         | 99.9         | 100                  |
| <b>41.4</b> | 99.9             | 99.6        | 99.8           | 98.4             | 99.8        | 92.7         | 91.0         | 99.9         | 99.8                 |
| <b>44.6</b> | 99.9             | 99.7        | 99.7           | 97.7             | 99.9        | 94.9         | 91.7         | 100          | 99.9                 |
| <b>41.7</b> | 99.2             | 98.5        | 99.2           | 96.4             | 99.8        | 93.2         | 90.5         | 100          | 99.9                 |

**[00118]** The MPSCs herein may provide a solution as an alternative to mesenchymal stromal cells (MSCs). Human MSCs exert immunosuppressive effects, demonstrate tri-lineage differentiation *in vitro*, and have been safely delivered to patients for a variety of indications, and have been approved to treat niche indications such as autoimmune-mediated perianal fistulas and Graft versus Host disease. However, widespread adoption of MSC-based therapies has been hindered by an inability to manufacture large batches of MSCs due to a population doubling limit of about 30-40 doublings before reaching cellular senescence.

**[00119]** The MPSCs herein have shown natural killer cell phenotypes as measured by FACS, *see* Table 3 below.

**[00120] Table 3. Expression of the markers showing natural killer cell phenotype**

| PD   | CD3 (-) | CD56 (+) | CD16 (+) |
|------|---------|----------|----------|
| 19.8 | 99.4    | 56.4     | 2.0      |
| 41.4 | 99.7    | 23.3     | 0.5      |
| 44.6 | 99.6    | 22.4     | 0.8      |
| 41.7 | 99.5    | 19.3     | 1.0      |

**[00121]** MPSCs expressed a variety of cell biomarkers including  $\beta$ -hCG, HLA-G, heat shock protein 90 (HSP90), and CDX2 immunocytochemically (**Figure 5A**). However, MPSCs did not express proliferation marker Ki-67, HSP70, tumor suppressor p53, and cell-cell fusion protein Syncytin (**Figure 5B**), supporting the concept that MPSCs stand at the first position of TE-differentiated trophoblasts. Specifically, MPSCs expressed HLA-A,B,C and surface and intracellular HLA-G by flow cytometry analysis (FACS) using different antibodies (**Figure 5C**; **Figure 5D**). However, they did not express HLA-DR.

**[00122]** **Figure 5D** are representative FACS images of HLA-G isoforms in MPSCs. Very few of all 7 isoforms detected at cell surface (upper left column) but 68.7% of all HLA-G 7 isoforms detected in permeabilized MPSCs (left lower column) by using Ab 4H84. While few of HLA-G G1 at cell surface (upper middle column) but 8.1% of HLA-G G1 (upper middle column) detected by Ab MEM-G/11. Similar few of HLA-G G1, G3, G5 detected at cell surface (upper right column) but none of HLA-G detected by Ab MEM-G9.

**[00123]** Human MPSCs Exhibit Immune Cell-Associated Biomarkers. The cells were characterized with immunocytochemistry and FACS analysis. The results showed that MPSCs expressed a variety of biomarkers associated with immune cells, including: cluster of differentiation (CD)56, CD16<sup>dim</sup>, inhibitory receptor KIR2DL4, CD11b, activating receptor NKp46, and CD10 of NK cells (**Figures 6A**); TCR, CD49f, ILT-4, CD3, CD4, CD8, CD44, CD90/Thy-1, CD44, and CD166 of T cells (**Figure 6B**); CD19 and CD141 of dendritic cells (**Figures 6C and 6D**); CD14 of macrophages (**Figure 6E**); Flt3L (**Figure 6F**) and CD34 of hematopoietic stem cells (**Figure 6G**); and CD38 of lymphocytes(**Figure 6G**). Subsequently, the expression of those biomarkers in MPSCs was analyzed with 8 independent cell lines, showing a similar pattern of NK and T cell biomarkers, wherein (CD16+CD56)<sup>+</sup> cells and CD107(+) cells showed the highest expression in the MPSCs. Biomarkers of NK cells and T cells occupy the most immune cells in MPSCs, while CD107(+)CD(16+56)(+) cells and CD8(+)CD(16+56)(+) cells occupied the most cell populations in MPSCs by FACS analysis.

**Example 4. A significant portion of MPSCs are monoclonal**

**[00124]** MPSC monoclonal cells were obtained from a MPSC culture. MPSCs were grown placed on an inverted microscope to record the cell type. The old medium was removed and cells were washed with sterile PBS. TRYPLE® solution was added to the MPSC culture. The cells were incubated at 37°C, 5% CO<sub>2</sub> for about 6 minutes. After incubation, the cells were separated. Culture media was added to the cells to stop the TRYPLE® reaction. The cells were collected, and a cell counter was used to calculate the number of cells. About 200 cells were removed and placed in a centrifuge tube. The media was replenished, and the cells were divided into 96 well plates with a multichannel pipette. The 96 well plates were grown at 37 °C, 5% CO<sub>2</sub>, and incubated for about 14 days. The media was changed every 2-3 days during this process. The old medium was washed and a TRYPLE® solution was added to the cells. After a short incubation, culture media was added to stop the TRYPLE® reaction, and the cells were moved to a 6 well plate and grown at 37 °C and 5% CO<sub>2</sub>. The culture media was changed every 2-3 days until they were sub-cultured into a 100mm dish and grown at 37 °C and 5% CO<sub>2</sub>. The media was changed every 2-3 days. The monoclonal cells were frozen when they reach 80-95% thickness. From donor ectopic tissue, the cells were expanded as wild-type passages with mixed cells or they were expanded into monoclonal cells. Monoclonal cells were expanded to provide multiple doses. For example, for every 1 million cells derived from donor ectopic tissue, about 125,000 monoclonal cells can be cultured. Each monoclonal cell has the potential for  $7 \times 10^{28}$  cells. At 100 million cells per dose, each monoclonal cell can make  $7 \times 10^{20}$  doses. Total potential from each ectopic tissue collected:  $125,000 \times 7 \times 10^{20}$  doses =  $8.8 \times 10^{25}$  doses. Each MPSC monoclonal cell can support a complete product cycle.

**[00125]** Every vial of 1M cells can potentially net about 130,000 monoclonal cells. See Table 4 below.

**[00126]** **Table 4. Monoclonal percentages among the MPSCs**

| Cell lines | Cells Seeded | Monoclonal Established | %     |
|------------|--------------|------------------------|-------|
| I          | 1600         | 203                    | 12.7% |
| II         | 800          | 119                    | 14.9% |
| III        | 400          | 50                     | 12.5% |
| Average    |              |                        | 13.3% |

**Example 5. MPSCs are pathogen-free**

**[00127]** Regardless whether source cells were infected or free from pathogen, the human MPSC cells obtained herein were pathogen-free. Nine cell lines of MPSCs were tested. PCR

evaluation was done to detect *Corynebacterium bovis*, *Corynebacterium* sp. (HAC2), EBV, HAdV, Hantaan Hantavirus, HCMV, Hepatitis A, Hepatitis B, Hepatitis C, HHV 6, HHV 8, HIV1, HIV2, HPV16, HPV18, HSV 1, HSV 2, HTLV 1, HTLV2, LCMV, *Mycoplasma* sp., Seoul Hantavirus, Sin Nombre Hantavirus, *Treponema pallidum*, VZV. All of the cell lines were found negative for all 25 pathogens in the h-IMPACT I panel.

**Example 6. Optimization HLA-G FACS Staining (Surface vs. Intracellular)**

**[00128]** MPSC wild type (WT) cell line 1 (MPSC1) was cultured in nutritional media + cell attachment substrate and passaged 12 times at 37 °C with 5% CO<sub>2</sub>. Fixed and permeabilized cells were prepared for cell surface and intracellular staining. Staining conditions are as follows in Table 5:

**[00129] Table 5:**

|                                                | Fixed | Permeabilized | HLA-G<br>Primary<br>Antibody                                                                                      | Dilution     | Secondary<br>Antibody | Dilution |
|------------------------------------------------|-------|---------------|-------------------------------------------------------------------------------------------------------------------|--------------|-----------------------|----------|
| <b>Cell Surface and Intracellular Staining</b> |       |               |                                                                                                                   |              |                       |          |
| 1                                              | YES   | YES           |                                                                                                                   |              |                       | 1:2000   |
| 2                                              | YES   | YES           | MEM-G/11<br>(Mouse/IgG1,<br>Invitrogen,<br>Cat. No.<br>MA1-19350,<br>1 mg/mL<br>solution, 2<br>µg/mL<br>dilution) | 1:25-<br>200 | Alexa - 488           | 1:2000   |

**[00130]** Primary antibody histogram results are shown below in Table 6 for 1:25, 1:50, 1:100, and 1:200 dilutions.

**[00131] Table 6.**

|   | Primary Antibody<br>Dilution | % Positive Cells HLA-<br>G 4H84 | % Positive Cells HLA-<br>G MEM-G/11 |
|---|------------------------------|---------------------------------|-------------------------------------|
| 1 | 1:25                         | 74.88                           | 1.53                                |
| 2 | 1:50                         | 75.96                           | 1.5                                 |
| 3 | 1:100                        | 72.87                           | 3.54                                |
| 4 | 1:200                        | 31.88                           | 2.32                                |

[00132] Primary antibody dot plot results (A-FL1 vs. SSC) are shown below in Table 7 for 1:25, 1:50, 1:100, and 1:200 dilutions.

[00133] **Table 7.**

|   | Primary Antibody Dilution | % Positive Cells |
|---|---------------------------|------------------|
| 1 | 1:25                      | 98.08            |
| 2 | 1:50                      | 96.75            |
| 3 | 1:100                     | 97.62            |
| 4 | 1:200                     | 86.44            |

[00134] Primary antibody dot plot results (FL1 vs. FL2) are shown below in Table 8 for 1:25, 1:50, 1:100, and 1:200 dilutions.

[00135] **Table 8:**

|   | Primary Antibody Dilution | % Positive Cells |
|---|---------------------------|------------------|
| 1 | 1:25                      | 90.16            |
| 2 | 1:50                      | 88.61            |
| 3 | 1:100                     | 79.29            |
| 4 | 1:200                     | 33.31            |

[00136] MPSC1 was cultured in Nutritional media + cell attachment substrate and passaged 7 times (19.8 population doublings) at 37 °C with 5% CO<sub>2</sub>. Fixed and permeabilized cells were prepared for cell surface and intracellular staining with HLA-G 4H84 antibody and HLA-G MEM-G/11 antibody. Cells fixed after surface staining were analyzed with HLA-G MEM-G/11 antibody. Results are shown below in Table 9.

[00137] **Table 9:**

| Primary Antibody Dilution             | % Positive Cells HLA-G 4H84 | % Positive Cells HLA-G MEM-G/11 |
|---------------------------------------|-----------------------------|---------------------------------|
| Cell surface + intracellular staining |                             |                                 |
| 1:50                                  | 95.06                       | 1.72                            |
| 1:100                                 | 79.1                        | -                               |
| Cells fixed after surface staining    |                             |                                 |
| 1:25                                  | -                           | 2.89                            |
| 1:50                                  | -                           | 1.36                            |
| 1:100                                 | -                           | 1.76                            |

[00138] HLA-G staining of MPSC 1 with FLA-G 4H86 dot plot results (FL1 vs. SSC) compared to controls are shown in Table 10:

**[00139] Table 10:**

| Primary Antibody Dilution | % Positive Cells |
|---------------------------|------------------|
| 1:50                      | 99.64            |

**Example 7. Generation of developmental cell banks and evaluation thereof**

**[00140]** Four extra-embryonic stem cell lines (for example, human trophoblast stem cells) were utilized as source cells: Mortal pluripotent stem cell line 1 (MPSC1), MPSC2, MPSC3, and MPSC4. Cell banks were developed by culturing the cell lines separately in nutritional media (*e.g.*, MESENCULT™ + cell attachment substrate). Subcultures of cells were seeded at densities between 3000 – 4000 cells/cm<sup>2</sup> and were cultured for three or four days. Endpoints of the study were 10 PD, 35 PD, 55 PD, and 70 PD. Thereafter, the phenotype was assessed by FACS characterization, MPSC/NK markers, and HLA-G. Additionally, functionality of the produced cells was assessed.

**Example 8. MPSC/NK production, Phenotype and Functionality Experiments***Mortal Pluripotent Stem Cell Line 1 (MPSC1)*

**[00141]** Mortal pluripotent stem cell line 1 (MPSC1) was utilized as source cells. The culture was seeded with 3000/4000 cells/cm<sup>2</sup>, cultured in nutritional media (*e.g.*, MESENCULT + cell attachment substrate), and expanded. Two cell banks (CB) were frozen down: CB2: 31.3 PD and CB3: 53.1 PD. Phenotypic and functional assays were conducted.

**[00142]** C1 characterization was as follows: MPSC1 was assessed for functionality (trilineage differentiation and secretome analysis).

**[00143]** C2 characterization was as follows: MPSC1 P13, 40.3 PD was assessed by FACS for MSC/NK markers and HLA-G for phenotype determination, and for functionality (trilineage differentiation and secretome analysis).

*Mortal Pluripotent Stem Cell Line 2*

**[00144]** Mortal pluripotent stem cell line 2 (MPSC2) was utilized as source cells. The culture was seeded with 3000/4000 cells/cm<sup>2</sup>, cultured in nutritional media (*e.g.*, MESENCULT™ + cell attachment substrate), and expanded. Three cell banks (CB) were frozen down: CB1: 8.1 PD; CB2: 29.3 PD; and CB3: 47.4 PD. Phenotypic and functional assays were conducted. Average doubling time (P4-P6) was 26.9 hours.

**[00145]** C1 characterization was as follows: MPSC2 passage 5 (P5), 11.2 passage doubling (PD) was assessed by FACS for MSC/NK markers and HLA-G for phenotype determination, and for functionality (trilineage differentiation and secretome analysis).

[00146] C2 characterization was as follows: MPSC2 P16, 36.9 PD was assessed by FACS for MSC/NK markers and HLA-G for phenotype determination, and for functionality (trilineage differentiation and secretome analysis).

*Mortal Pluripotent Stem Cell Line 3*

[00147] Mortal pluripotent stem cell line 3 (MPSC3) was utilized as source cells. The culture was seeded with 3000/4000 cells/cm<sup>2</sup>, cultured in nutritional media (*e.g.*, MESENCULT™ + cell attachment substrate), and expanded. Three cell banks (CB) were frozen down: CB1: 8.1 PD; CB2: 25.5 PD; and CB3: 37.3 PD. Phenotypic and functional assays were conducted. Average doubling time (P4-P6) was 32.7 hours.

[00148] C1 characterization was as follows: MPSC3 passage 5 (P5), 10.2 passage doubling (PD) was assessed by FACS for MSC/NK markers and HLA-G for phenotype determination, and for functionality (trilineage differentiation and secretome analysis).

[00149] C2 characterization was as follows: MPSC3 P18, 33.3 PD was assessed by FACS for MPSC/NK markers and HLA-G for phenotype determination, and for functionality (trilineage differentiation and secretome analysis).

*Mortal Pluripotent Stem Cell Line 4*

[00150] Mortal pluripotent stem cell line (MPSC4) was utilized as source cells. The culture was seeded with 3000/4000 cells/cm<sup>2</sup>, cultured in nutritional media (*e.g.*, MESENCULT™ + cell attachment substrate), and expanded for a total of about 6.5 PD. The cells were either frozen or characterized by FACS analysis for mesenchymal stem cell (MSC)/natural killer NK markers and HLA-G. Average doubling time from P4-P5 was approximately 43.7 hours.

*Growth Curves*

[00151] Growth curves of the four MPSC cell lines is shown in **FIG. 7**. MPSC1 for this experiment underwent 2 freeze/thaw cycles that likely decreased the maximum number of PD in culture.

[00152] Results from a second experiment of the growth curves and collections of cell banks (CB) 1, 2, 3, and 4 for MPSC1, MPSC2, MPSC3, and MPSC4 were obtained. Doubling of MPSC4 was slow and the experiment for that cell line was discontinued.

| PD  | MPSC1 | MPSC2 | MPSC3 |
|-----|-------|-------|-------|
| CB1 | 13.5  | 8.1   | 8.1   |
| CB2 | 31.3  | 29.3  | 25.5  |
| CB3 | 53.1  | 47.4  | 37.3  |

| PD  | MPSC1 | MPSC2 | MPSC3 |
|-----|-------|-------|-------|
| CB4 | 59.7  | 55.5  | 45.2  |

[00153] Phenotypic characterization was conducted at the following passage doubling (PD) times for each cell line:

| PD    | Collection 1<br>(C1) | Collection 2<br>(C2) | Collection 3<br>(C3) | Collection 4<br>(C4) |
|-------|----------------------|----------------------|----------------------|----------------------|
| MPSC1 | -                    | 40.3                 | 58.3                 | 67.4                 |
| MPSC2 | 11.2                 | 36.9                 | -                    | -                    |
| MPSC3 | 10.3                 | 33.3                 | -                    | -                    |
| MPSC4 | 5.1                  | -                    | -                    | -                    |

[00154] Cell surface antigen expression of MPSC/NK markers and HLA-G were assessed via FACS. Also assessed were adherence to plastic in standard culture conductions and multipotent differentiation potential into osteoblasts, adipocytes, and chondroblasts.

|            | POSITIVE MARKERS<br>( $\geq 95\%$ +) | NEGATIVE MARKERS<br>( $\leq 2\%$ +)   |
|------------|--------------------------------------|---------------------------------------|
| MPSC PANEL | CD29                                 | CD4                                   |
|            | CD44                                 | CD14 (monocytes, macrophages)         |
|            | CD73                                 | CD19 (B cells)                        |
|            | CD90                                 | CD34 (hematopoietic progenitors, ECs) |
|            | CD105                                | CD45 (leukocytes)                     |
|            | CD146                                | HLA-DR                                |
|            | CD166                                |                                       |
|            | HLA-A, B, C                          |                                       |
| NK PANEL   | CD16                                 | CD3                                   |
|            | CD56                                 |                                       |
|            | CD107                                |                                       |
| HLA-G      | HLA-G                                |                                       |

[00155] FACS Characterization of MPSC Markers, HLA-G for C1 and C2 are as follows:

| MPSC Markers   | PD | Negative Markers |         |         |         |         |            |
|----------------|----|------------------|---------|---------|---------|---------|------------|
| Media/ Coating |    | CD4(-)           | CD14(-) | CD19(-) | CD34(-) | CD45(-) | HLA-DR (-) |
| C1             |    |                  |         |         |         |         |            |

| MPSC Markers                                      | PD   | Negative Markers |         |         |         |         |            |
|---------------------------------------------------|------|------------------|---------|---------|---------|---------|------------|
| Media/ Coating                                    |      | CD4(-)           | CD14(-) | CD19(-) | CD34(-) | CD45(-) | HLA-DR (-) |
| <b>MPSC4</b><br><b>MESENCULT™ +</b><br><b>CAS</b> | 5.1  | 99.5             | 98.8    | 99.5    | 99.3    | 99.6    | 99.5       |
| <b>MPSC2</b><br><b>MESENCULT™ +</b><br><b>CAS</b> | 11.2 | 99.9             | 99.9    | 99.8    | 100     | 99.7    | 99.8       |
| <b>MPSC3</b><br><b>MESENCULT™ +</b><br><b>CAS</b> | 10.3 | 99.8             | 99.9    | 99.9    | 99.8    | 99.9    | 99.7       |
| <b>C2</b>                                         |      |                  |         |         |         |         |            |
| <b>MPSC1</b><br><b>MESENCULT™ +</b><br><b>CAS</b> | 40.3 | 98.8             | 99.2    | 99.5    | 99.6    | 99.3    | 99.7       |
| <b>MPSC2</b><br><b>MESENCULT™ +</b><br><b>CAS</b> | 36.9 | 99.6             | 99.5    | 99.5    | 99.6    | 99.5    | 99.6       |
| <b>MPSC3</b><br><b>MESENCULT™ +</b><br><b>CAS</b> | 33.3 | 98.8             | 99.0    | 99.5    | 99.4    | 99.5    | 99.5       |
| <b>C3</b>                                         |      |                  |         |         |         |         |            |
| <b>MPSC1</b><br><b>MESENCULT™ +</b><br><b>CAS</b> | 58.3 | 98.9             | 99.0    | 99.3    | 99.4    | 99.2    | 99.4       |
| <b>C4</b>                                         |      |                  |         |         |         |         |            |
| <b>MPSC1</b><br><b>MESENCULT™ +</b><br><b>CAS</b> | 67.4 | 98.5             | 97.4    | 98.4    | 98.4    | 98.3    | 98.6       |

| MPSC Markers   | PD | Positive Markers |          |          |          |
|----------------|----|------------------|----------|----------|----------|
| Media/ Coating |    | CD29 (+)         | CD44 (+) | CD73 (+) | CD90 (+) |

| <b>C1</b>                     |      |      |      |      |      |
|-------------------------------|------|------|------|------|------|
| <b>MPSC4 MESENCULT™ + CAS</b> | 99.9 | 73.9 | 99.0 | 51.4 | 99.9 |
| <b>MPSC2 MESENCULT + CAS</b>  | 100  | 98.1 | 90.3 | 98.2 | 100  |
| <b>MPSC3 MESENCULT™ + CAS</b> | 99.8 | 92.8 | 97.7 | 94.7 | 99.8 |
| <b>C2</b>                     |      |      |      |      |      |
| <b>MPSC1 MESENCULT™ + CAS</b> | 99.9 | 98.7 | 99.9 | 99.8 | 99.9 |
| <b>MPSC2 MESENCULT™ + CAS</b> | 99.9 | 99.1 | 99.1 | 99.1 | 99.9 |
| <b>MPSC3 MESENCULT™ + CAS</b> | 99.9 | 98.0 | 99.9 | 98.9 | 99.9 |
| <b>C3</b>                     |      |      |      |      |      |
| <b>MPSC1 MESENCULT™ + CAS</b> | 58.3 | 99.9 | 99.4 | 99.9 | 99.9 |
| <b>C4</b>                     |      |      |      |      |      |
| <b>MPSC1 MESENCULT™ + CAS</b> | 67.4 | 98.5 | 65.6 | 97.6 | 95.4 |

| MPSC Markers              | PD   | Positive     |              |              |                   |              |
|---------------------------|------|--------------|--------------|--------------|-------------------|--------------|
| Media/ Coating            |      | CD105<br>(+) | CD146<br>(+) | CD166<br>(+) | HLA-<br>A,B,C (+) | HLA-G<br>(+) |
| C1                        |      |              |              |              |                   |              |
| MPSC4 MESENCULT™<br>+ CAS | 39.2 | 65.0         | 99.9         | 93.4         | 98.6              | 39.2         |
| MPSC2 MESENCULT™<br>+ CAS | 97.9 | 72.3         | 99.8         | 99.9         | 72.7              | 97.9         |
| MPSC3 MESENCULT™<br>+ CAS | 100  | 99.9         | 99.9         | 99.9         | 95.4              | 100          |
| C2                        |      |              |              |              |                   |              |
| MPSC1 MESENCULT™          | 93.2 | 95.9         | 99.9         | 99.9         | 97.3              | 93.2         |

| <b>MPSC Markers</b>               | <b>PD</b> | <b>Positive</b>      |                      |                      |                      |                      |
|-----------------------------------|-----------|----------------------|----------------------|----------------------|----------------------|----------------------|
| <b>Media/ Coating</b>             |           | <b>CD105<br/>(+)</b> | <b>CD146<br/>(+)</b> | <b>CD166<br/>(+)</b> | <b>HLA-A,B,C (+)</b> | <b>HLA-G<br/>(+)</b> |
| <b>+ CAS</b>                      |           |                      |                      |                      |                      |                      |
| <b>MPSC2 MESENCULT™<br/>+ CAS</b> | 97.6      | 99.0                 | 99.9                 | 99.6                 | <b>97.5</b>          | 97.6                 |
| <b>MPSC3 MESENCULT™<br/>+ CAS</b> | 99.9      | 99.9                 | 99.9                 | 99.7                 | <b>98.7</b>          | 99.9                 |
| <b>C3</b>                         |           |                      |                      |                      |                      |                      |
| <b>MPSC1 MESENCULT™<br/>+ CAS</b> | 58.3      | 73.7                 | 98.7                 | 99.9                 | 99.9                 | 97.4                 |
| <b>C4</b>                         |           |                      |                      |                      |                      |                      |
| <b>MPSC1 MESENCULT™<br/>+ CAS</b> | 67.4      | 68.0                 | 93.7                 | 98.8                 | 95.4                 | 83.0                 |

| NK Markers                | PD   | Negative Markers | Positive Markers |          |            |                 |
|---------------------------|------|------------------|------------------|----------|------------|-----------------|
| Media/ Coating            |      | CD3 (-)          | CD16 (+)         | CD56 (+) | CD107* (+) | CD107 ICS** (+) |
| C1                        |      |                  |                  |          |            |                 |
| MPSC4 MESENCULT™<br>+ CAS | 5.1  | N/A              | N/A              | 33.6     | 11.7       | 81.0            |
| MPSC2 MESENCULT™<br>+ CAS | 11.2 | 99.9             | 0.09             | 61.5     | 13.2       | 91.2            |
| MPSC3 MESENCULT™<br>+ CAS | 10.3 | N/A              | N/A              | 0.4      | 17.2       | 97.9            |
| C2                        |      |                  |                  |          |            |                 |
| MPSC1 MESENCULT™<br>+ CAS | 40.3 | 99.8             | 0.4              | 14.4     | 6.8        | 97.1            |
| MPSC2 MESENCULT™<br>+ CAS | 36.9 | 99.6             | 1.0              | 43.0     | 8.4        | 91.8            |

| <b>NK Markers</b>                 | <b>PD</b>   | <b>Negative Markers</b> | <b>Positive Markers</b> |                 |                   |                        |
|-----------------------------------|-------------|-------------------------|-------------------------|-----------------|-------------------|------------------------|
| <b>Media/ Coating</b>             |             | <b>CD3 (-)</b>          | <b>CD16 (+)</b>         | <b>CD56 (+)</b> | <b>CD107* (+)</b> | <b>CD107 ICS** (+)</b> |
| <b>MPSC3 MESENCULT™<br/>+ CAS</b> | <b>33.3</b> | 99.4                    | 1.1                     | <b>1.4</b>      | <b>8.6</b>        | <b>99.0</b>            |
| <b>C3</b>                         |             |                         |                         |                 |                   |                        |
| <b>MPSC1 MESENCULT™<br/>+ CAS</b> | 58.3        | 99.5                    | 1.3                     | <b>16.8</b>     | <b>5.07</b>       | <b>99.4</b>            |
| <b>C4</b>                         |             |                         |                         |                 |                   |                        |
| <b>MPSC1 MESENCULT™<br/>+ CAS</b> | 67.4        | 96.8                    | 2.4                     | <b>4.2</b>      | <b>9.74</b>       | <b>84.1</b>            |

**[00156]**      Functionality: Trilineage differentiation was assessed using the following differentiation protocols:

|          | <b>Differentiation</b> | <b>Media</b>                                                                              | <b>Culture Conditions</b>                                                                                                                                                                        | <b>Duration</b>                                                                         | <b>Staining</b>                               |
|----------|------------------------|-------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------|-----------------------------------------------|
| <b>1</b> | Adipogenesis           | MESENCULT™<br>Adipogenic<br>Differentiation<br>Kit,<br>STEMCELL<br>Technologies<br>#05412 | <b>FORMAT:</b> 6/12-well<br>plates, CAS-coated<br><b>SEEDING DENSITY:</b><br>4,000 cells/cm <sup>2</sup><br><b>DIFFERENTIATION:</b><br>at 95% confluency<br><b>MEDIA CHANGE:</b><br>every 3 days | C1: T1 =<br>5.5/7<br>weeks<br>C2: T1 =<br>4 weeks<br>T2 = 6<br>weeks<br>T3 = 8<br>weeks | <b>Oil Red O</b><br>(fixed<br>cells)          |
| <b>2</b> | Osteogenesis           | MESENCULT™<br>Osteogenic<br>Differentiation<br>Kit,<br>STEMCELL<br>Technologies<br>#05465 | <b>FORMAT:</b> 6/12-well<br>plates, CAS-coated<br><b>SEEDING DENSITY:</b><br>4,000 cells/cm <sup>2</sup><br><b>DIFFERENTIATION:</b><br>at 95% confluency                                         | C1: T1 =<br>3 weeks<br>C2: T1 =<br>3 weeks<br>T2 = 4<br>weeks<br>T3 = 5<br>weeks        | <b>Alizarin<br/>Red S</b><br>(fixed<br>cells) |

|          |                |                                                                                                     |                                                                                                                                                                                |                            |                                                                                             |
|----------|----------------|-----------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------|---------------------------------------------------------------------------------------------|
|          |                |                                                                                                     | <b>MEDIA CHANGE:</b><br>every 3 days                                                                                                                                           |                            |                                                                                             |
| <b>3</b> | Chondrogenesis | MESENCULT™-<br>ACF<br>Chondrogenic<br>Differentiation<br>Kit,<br>STEMCELL<br>Technologies<br>#05455 | <b>FORMAT:</b> 15 mL<br>conical tubes<br><b>SEEDING DENSITY:</b><br>cell pellet 300,000 cells<br><b>DIFFERENTIATION:</b><br>at seeding<br><b>MEDIA CHANGE:</b><br>every 3 days | C1, C2:<br>T1 = 3<br>weeks | <b>Alcian<br/>Blue</b><br>(fixed<br>pellets,<br>paraffin-<br>embedded<br>and<br>sectioned)* |

**[00157]** MPSC3 showed no differentiation into adipocytes after 7 weeks in adipogenic differentiation medium. In contrast, MPSC1 and MPSC2 demonstrated the ability to differentiate into adipocytes at 5.5 weeks and 7 weeks, respectively. Data not shown.

**[00158]** At week 3, MPSC1, MPSC2, and MPSC3 C1 cells demonstrated osteogenesis utilizing alizarin red staining compared to controls. Data not shown.

#### **Example 9. Differentiation induced by hypoxia**

**[00159]** Extra embryonic stem cell lines (e.g., human trophoblastic stem cells) were grown in a nutritional media (e.g., MESENCULT™ with a cell attachment substrate) until confluency was reached (e.g., from about 3000 cells/cm<sup>2</sup> to about 9000 cells/cm<sup>2</sup>, or about 6000 cells/cm<sup>2</sup>). Cells were washed and the media was replaced without Supplement. Hypoxia was induced in a chamber (e.g., culture for about 24 hours in a 2% O<sub>2</sub> gas mixture). Medium was collected and frozen until use. Media from all three cell lines was tested using a QUANTIBODY™ Human Kiloplex Array (RAYBIOTECH™ Life, Inc.) to quantitatively analyze 1000 proteins. Experiments for MPSC1 and MPSC2 were repeated. Briefly, samples were processed and analyte concentration (pg/mL) were determined and compared to standard curves. Data was determined as % samples below the Limit of Detection (LOD), % samples above LOD, but <3×LOD, % samples in Best Confidence Interval, and % samples above maximum.

**[00160]** Population Doublings and Doubling Time of MPSCs are as follows. DT hours (hrs.): average doubling time calculated over 3 consecutive passages upon thawing, excluding the first 2 passages immediately after thaw to allow for complete cell recovery. \*\* PD: maximum number of population doublings achieved in culture for the corresponding culture condition.

|    | Cell Line | Case Study | Culture Conditions                     | DT (hrs)* | PD** |
|----|-----------|------------|----------------------------------------|-----------|------|
| 1  | MPSC5     | PR001-R2   | $\alpha$ -MEM + STEMULATE              | 26.0      | 53.1 |
| 2  |           |            | Rooster Nourish MSC + CellBind Surface | 21.0      | 59.2 |
| 3  |           |            | MESENCULT™ + CAS Coating               | 27.0      | 75.9 |
| 4  | MPSC1 WT  | PR002-R1   | $\alpha$ -MEM + STEMULATE              | 26.0      | 67.6 |
| 5  |           |            | MESENCULT™ + CAS Coating               | 21.5      | 85.2 |
| 6  |           |            | MESENCULT™ NO CAS Coating              | 24.7      | 75.6 |
| 7  | MPSC1 WT  | PR002-R2   | MESENCULT™ + CAS Coating               | 22.5      | 76.8 |
| 8  | MPSC1 WT  | PR002-R4   | MESENCULT™ + CAS Coating               | 22.9      | 74.3 |
| 9  | MPSC6     | PR003      | MESENCULT™ + CAS Coating               | 40.7      | N/A  |
| 10 | MPSC7     | PR004      | MESENCULT™ + CAS Coating               | 40.2      | N/A  |
| 11 | MPSC4     | PR005      | MESENCULT™ + CAS Coating               | 43.7      | N/A  |
| 12 | MPSC2     | PR006      | MESENCULT™ + CAS Coating               | 26.9      | 56.4 |
| 13 | MPSC3     | PR007      | MESENCULT™ + CAS Coating               | 32.7      | 46.3 |

[00161] FACS characterization of C1 cells for MPSC negative markers is as follows:

|    | MPSC Negative Markers |          |          |            |
|----|-----------------------|----------|----------|------------|
|    | PD                    | CD19 (-) | CD45 (-) | HLA-DR (-) |
| 1  | 22.9                  | 99.9     | 99.9     | 99.2       |
| 2  | 22.2                  | 99.9     | 99.8     | 99.7       |
| 3  | + 5.5                 | 99.9     | 99.9     | 99.9       |
| 4  | +5.2                  | 99.8     | 99.7     | 99.8       |
| 5  | 19.0                  | 99.9     | 99.9     | 99.9       |
| 6  | 18.1                  | 99.8     | 99.8     | 99.8       |
| 7  | 19.8                  | 99.9     | 98.6     | 99.6       |
| 8  | + 5.5                 | 99.9     | 99.8     | 99.9       |
| 9  | + 3.9                 | 99.9     | 99.7     | 99.8       |
| 10 | 5.1                   | 99.5     | 99.6     | 99.5       |

|    | <b>MPSC Negative Markers</b> |                 |                 |                   |
|----|------------------------------|-----------------|-----------------|-------------------|
|    | <b>PD</b>                    | <b>CD19 (-)</b> | <b>CD45 (-)</b> | <b>HLA-DR (-)</b> |
| 11 | <b>11.2</b>                  | 99.8            | 99.7            | 99.8              |
| 12 | <b>10.3</b>                  | 99.9            | 99.9            | 99.7              |
| 13 | <b>22.9</b>                  | 99.9            | 99.9            | 99.2              |

**[00162]** FACS characterization of C1 cells for MPSC positive markers is as follows:

|    | <b>MPSC Positive Markers</b> |                 |                 |                  |                  |                  |                      |                  |
|----|------------------------------|-----------------|-----------------|------------------|------------------|------------------|----------------------|------------------|
|    | <b>PD</b>                    | <b>CD44 (+)</b> | <b>CD90 (+)</b> | <b>CD105 (+)</b> | <b>CD146 (+)</b> | <b>CD166 (+)</b> | <b>HLA-A,B,C (+)</b> | <b>HLA-G (+)</b> |
| 1  | <b>22.9</b>                  | 98.9            | 100.0           | 100.0            | 98.2             | 100.0            | 100.0                | N/A              |
| 2  | <b>22.2</b>                  | 99.1            | 99.8            | 100.0            | 98.6             | 100.0            | 100.0                | N/A              |
| 3  | <b>+ 5.5</b>                 | 99.6            | 99.9            | 99.9             | 99.9             | 99.9             | 99.9                 | N/A              |
| 4  | <b>+5.2</b>                  | 99.2            | 99.9            | 99.9             | 99.9             | 99.9             | 99.9                 | N/A              |
| 5  | <b>19.0</b>                  | 99.7            | 99.9            | 99.8             | 98.7             | 99.8             | 99.9                 | N/A              |
| 6  | <b>18.1</b>                  | 98.6            | 99.9            | 97.4             | 92.9             | 99.9             | 99.9                 | N/A              |
| 7  | <b>19.8</b>                  | 99.7            | 99.8            | 96.6             | 92.1             | 99.9             | 100                  | 95.1             |
| 8  | <b>+ 5.5</b>                 | 99.6            | 100.0           | 100.0            | 99.5             | 99.9             | 99.7                 | 97.5             |
| 9  | <b>+ 3.9</b>                 | 99.4            | 99.2            | 99.9             | 91.0             | 99.9             | 99.2                 | 98.6             |
| 10 | <b>5.1</b>                   | 73.9            | 51.4            | 39.2             | 65.0             | 99.9             | 93.4                 | 98.6             |
| 11 | <b>11.2</b>                  | 98.1            | 98.2            | 97.9             | 72.3             | 99.8             | 99.9                 | 72.7             |
| 12 | <b>10.3</b>                  | 92.8            | 94.7            | 100              | 99.9             | 99.9             | 99.9                 | 95.4             |
| 13 | <b>22.9</b>                  | 98.9            | 100.0           | 100.0            | 98.2             | 100.0            | 100.0                | N/A              |

**[00163]** FACS characterization of C1 cells for NK positive markers is as follows:

|   | <b>NK Markers</b> |                        |                         |                 |
|---|-------------------|------------------------|-------------------------|-----------------|
|   |                   | <b>Negative Marker</b> | <b>Positive Markers</b> |                 |
|   | <b>PD</b>         | <b>CD3 (-)</b>         | <b>CD56 (+)</b>         | <b>CD16 (+)</b> |
| 1 | <b>22.9</b>       | 99.8                   | <b>0.6</b>              | 0.0             |
| 2 | <b>22.2</b>       | 99.9                   | <b>2.7</b>              | 0.2             |
| 3 | <b>+ 5.5</b>      | 99.9                   | <b>54.0</b>             | 0.06            |
| 4 | <b>+5.2</b>       | 99.9                   | <b>47.8</b>             | 0.1             |

|    | NK Markers   |                 |                  |                 |
|----|--------------|-----------------|------------------|-----------------|
|    |              | Negative Marker | Positive Markers |                 |
| 5  | <b>19.0</b>  | 99.9            | <b>68.7</b>      | 0.1             |
| 6  | <b>18.1</b>  | 99.9            | <b>59.0</b>      | 0.3             |
| 7  | <b>19.8</b>  | 99.4            | <b>56.4</b>      | 98.0            |
| 8  | <b>+ 5.5</b> | 99.7            | <b>68.1</b>      | 0.4             |
| 9  | <b>+ 3.9</b> | 99.8            | <b>25.5</b>      | <b>0.7</b>      |
| 10 | <b>5.1</b>   | <i>N/A</i>      | <b>33.6</b>      | <i>N/A</i>      |
| 11 | <b>11.2</b>  | 99.9            | <b>61.5</b>      | <i>0.1</i>      |
| 12 | <b>10.3</b>  | <i>N/A</i>      | <b>0.4</b>       | <i>N/A</i>      |
| 13 | <b>22.9</b>  | <b>CD3 (-)</b>  | <b>CD56 (+)</b>  | <b>CD16 (+)</b> |

**[00164]** MPSC differentiation into pancreatic progenitor cells and neural stem cells was determined. Samples were collected to perform FACS analysis and mRNA analysis at 3 different concentrations and at 3 different time points.

| Procedure             | Pancreatic Progenitor Cells                            | Neural Stem Cells                                                 |
|-----------------------|--------------------------------------------------------|-------------------------------------------------------------------|
| Differentiation Media | MEM $\alpha$ + STEMULATE + Human bFGF 10 ng/mL         | MEM $\alpha$ + STEMULATE + all-trans retinoic acid, RA 10 $\mu$ M |
| Differentiation Time  | 24 hrs                                                 | 24 hrs                                                            |
| Markers FACS (+)      | HLA-G, b-HCG, CDX2, PDX-1, FOXA2, SOX9                 | RAR-b, N-CAD, SOX2, NESTIN, PAX6                                  |
| Markers FACS (-)      | CD34, CD45                                             | CD90, CD44                                                        |
| Comments              | Test 3 different bFGF concentrations                   | Test 3 different RA concentrations                                |
|                       | Collect samples for analysis 3 timepoints (24-72 hrs.) | Collect samples for analysis 3 timepoints (24-72 hrs.)            |
|                       | Collect samples for mRNA analysis                      | Collect samples for mRNA analysis                                 |
|                       | Perform differentiation at different PD                | Perform differentiation at different PD                           |
|                       | Antibodies and specific staining SOPs available        | Antibodies and specific staining SOPs available                   |

**Example 10. Generation of immunosuppressive cells and IDO secretion evaluation**

[00165] Three extra embryonic stem cell lines (for example, human trophoblast stem cells) were utilized as source cells: mortal pluripotent stem cell line 1 (MPSC1) P5 cells, MPSC2 P8 cells, and MPSC3 P8 cells. Cells were cultured separately in nutritional media (*e.g.*, MESENCULT™ + cell attachment substrate). Subcultures of cells were seeded at a density of about 5,000 cells/cm<sup>2</sup>, cultured for about three days, treated with interferon gamma (IFN- $\gamma$ ) at 0 ng/mL (control), about 20 ng/mL, about 50 ng/mL, or about 100 ng/mL for 24 hours. Thereafter, the cells and supernatant were collected. Immunosuppressive capabilities of the resulting MPSC cells were assessed via ELISA.

[00166] Indoleamine 2,3-dioxygenase (IDO) secretion upon IFN- $\gamma$  stimulation was assessed. **FIG. 8A** illustrates the standard curve for the assay. **FIG. 8B** illustrates the results of the three cell lines at the various concentrations of IFN- $\gamma$  stimulation on IDO secretion compared to control. MPSCs primed with IFN- $\gamma$  were not found to statistically increase IDO secretion.

**Example 11. Generation of immunosuppressive cells and kynurenine secretion evaluation**

[00167] Human trophoblastic stem cell line 1 (MPSC1) P5 cells were cultured in nutritional media (*e.g.*, MESENCULT™ + cell attachment substrate). Subcultures of cells were seeded at a density of about 5,000 cells/cm<sup>2</sup>, cultured for about three days, treated with interferon gamma (IFN- $\gamma$ ) at 0 ng/mL (control), about 20 ng/mL, about 50 ng/mL, or about 100 ng/mL for 24, 48, and 72 hours. Thereafter, the cells and supernatant were collected.

[00168] Kynurenine secretion upon IFN- $\gamma$  stimulation was assessed. **FIG. 9A** illustrates the standard curve for the assay. **FIG. 9B** illustrates the results of the effect of IFN- $\gamma$  stimulation on Kynurenine secretion at the three different concentrations at 24, 48, and 72 hours compared to control and media alone. MPSCs cells primed with IFN- $\gamma$  were not found to statistically increase Kynurenine secretion.

**Example 12. Generation of immunosuppressive cells and IL-2 secretion evaluation**

[00169] Mortal pluripotent stem cell line 1 (MPSC1) P5 cells were cultured in nutritional media (*e.g.*, MESENCULT™ + cell attachment substrate). Jurkat cells (about 100,000 cells/mL) were activated with 1  $\mu$ g/mL GIBCO® Phytohemagglutinin, M form (PHA-M) + 50 ng/mL Phorbol 12-myristate 13-acetate (PMA) for 24 hours.

[00170] Co-cultures of the MPSC1 cells and Jurkat cells were established. Samples were (1) MPSC1 alone, (2) MPSC1 + resting Jurkat cells, (3) MPSC1 + activated Jurkat cells, and (4) activated Jurkat cells alone. MPSCs were seeded at a density of from about 2000 to about 3000

cells/cm<sup>2</sup>. Jurkat cells were seeded at a density of from about 50,000 to about 500,000 cells/well. Cells were co-cultured for 24 or 48 hours, and supernatant collected. IL-2 secretion was assessed via ELISA. **FIG. 10A** illustrates the standard curve for the assay. **FIG. 10B** illustrates the results of the effect of IFN- $\gamma$  stimulation on IL-2 secretion at 24 and 48 hours. Coculture of MPSC1 and activated Jurkat cells induced the highest IL-2 secretion. Dose dependent increases were observed. **FIG. 10C** illustrates the effect of MPSCs seeding density of about 3000 cells/cm<sup>2</sup> at 24 hours of coculture compared to controls. Dose dependent increases were observed. **FIG. 10D** illustrates the effect of MPSCs seeding density of about 2000 cells/cm<sup>2</sup> at 24 hours of coculture compared to controls. Dose dependent increases were observed. **FIG. 10E** illustrates the effect of MPSCs seeding density of about 3000 cells/cm<sup>2</sup> at 48 hours of coculture compared to controls. Dose dependent increases were observed. **FIG. 10F** illustrates the effect of MPSCs seeding density of about 2000 cells/cm<sup>2</sup> at 48 hours of coculture compared to controls. Dose dependent increases were observed. MPSCs increased, rather than decreased, IL-2 secretion by activated Jurkat cells. FACS phenotype of the samples was determined and the results at 24 and 48 hours (hrs) are provided below.

|                              | Sample    | % M2 | Mean FL M2 |
|------------------------------|-----------|------|------------|
| <b>Control</b>               | Unstained | 0.05 | 338.6      |
|                              | IgG1      | 0.11 | 132.38     |
|                              | IgG2A     | 0.11 | 290.3      |
|                              | HLA-DR    | 0.12 | 399.6      |
|                              | HLA-I     | 99.9 | 926.5      |
| <b>IFN-gamma<br/>24 hrs.</b> | Unstained | 0.11 | 122.2      |
|                              | IgG1      | 0.1  | 269.5      |
|                              | IgG2A     | 0.08 | 441.1      |
|                              | HLA-DR    | 1.05 | 346.83     |
|                              | HLA-I     | 99.8 | 1767.3     |
| <b>IFN-gamma</b>             | Unstained | 0.13 | 223.3      |

|                |        |      |        |
|----------------|--------|------|--------|
| <b>48 hrs.</b> | IgG1   | 0.12 | 166.61 |
|                | IgG2A  | 0.13 | 591.4  |
|                | HLA-DR | 2.69 | 550.0  |
|                | HLA-I  | 99.8 | 2614.4 |

**Example 13. Differentiation of stem cells into pancreatic progenitor cells (PPC) or neural stem cells (NSC)**

*MPSC1*

**[00171]** Stem cells (*e.g.*, MPSC1 P4) at approximately  $1 \times 10^6$  cells were thawed in nutritional media (*e.g.*, MESENCULT™ + cell attachment substrate, or MEM-alpha + STEMULATE). The cells were then expanded at P5: 4000 cells/cm<sup>2</sup> MESENCULT™ + cell attachment substrate or 4000 cells/cm<sup>2</sup> MEM-alpha + STEMULATE. PPC or NSC differentiation began at P6. Culture and differentiation conditions are as follows:

|                                                                                                                             |
|-----------------------------------------------------------------------------------------------------------------------------|
| <b>Culture Conditions</b>                                                                                                   |
| MESENCULT™ + cell attachment substrate: 10,000 cells/cm <sup>2</sup><br>MEM-alpha + STEMULATE: 10,000 cells/cm <sup>2</sup> |
| <b>Differentiation</b>                                                                                                      |
| Completed after about 24 hours.<br>FACS and Immunofluorescence (IF) characterization.                                       |

**[00172]** Cells were evaluated for adhesion to microcarriers and suspension expansion. Expansion occurred in a 100 mL bioreactor.

*MPSC2*

**[00173]** Stem cells (*e.g.*, MPSC2 P4) at approximately  $1 \times 10^6$  cells were thawed in nutritional media (*e.g.*, MESENCULT™ + cell attachment substrate, or MEM-alpha + STEMULATE). The cells were then expanded at P5: 5000 cells/cm<sup>2</sup> MESENCULT™ + cell attachment substrate or 5000 cells/cm<sup>2</sup> MEM-alpha + STEMULATE. PPC or NSC differentiation began at P6. Culture and differentiation conditions are as follows:

|                                                                                                                             |
|-----------------------------------------------------------------------------------------------------------------------------|
| <b>Culture Conditions</b>                                                                                                   |
| MESENCULT™ + cell attachment substrate: 10,000 cells/cm <sup>2</sup><br>MEM-alpha + STEMULATE: 10,000 cells/cm <sup>2</sup> |
| <b>Differentiation</b>                                                                                                      |
| Completed after about 24 hours.                                                                                             |

|                                                    |
|----------------------------------------------------|
| FACS and Immunofluorescence (IF) characterization. |
|----------------------------------------------------|

*Differentiation Experimental Setup*

|                       | <b>Pancreatic Progenitor Cells</b>                                                                                        | <b>Neural Stem Cells</b>                                                                                                                        |
|-----------------------|---------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------|
| Differentiation Media | 1. MEM $\alpha$ + STEMULATE + Human basic fibroblast growth factor (bFGF) 10 ng/mL<br>2. MESENCULT™ + Human bFGF 10 ng/mL | 1. MEM $\alpha$ + STEMULATE + all-trans retinoic acid (RA) 10 $\mu$ M<br>2. MESENCULT™ + all-trans retinoic acid, retinoic acid (RA) 10 $\mu$ M |
| Differentiation Time  | 24 hrs.                                                                                                                   | 24 hrs.                                                                                                                                         |
| Markers FACS (+)      | HLA-G, beta-HCG, CDX2, PDX-1, FOXA2, SOX9                                                                                 | RAR-beta, N-CAD, SOX2, NESTIN, PAX6, HLA-G                                                                                                      |
| Markers FACS (-)      | CD34, CD45                                                                                                                | CD90, CD44, CDX2                                                                                                                                |
| Assays                | FACS, immunofluorescence (beta-HCG <sup>**</sup> )                                                                        | FACS, immunofluorescence (RAR-beta)                                                                                                             |
|                       | RT-PCR                                                                                                                    | RT-PCR                                                                                                                                          |

*Neural Stem Cell FACS Characterization*

*MPSC1 Culture Condition 1: Neural Stem Cell Markers*

| <b>MPSC1</b> | <b>Marker</b> | <b>STEMULATE Control</b>        | <b>STEMULATE + RA</b>           |
|--------------|---------------|---------------------------------|---------------------------------|
|              |               | <b>% + cells G2<sup>+</sup></b> | <b>% + cells G2<sup>+</sup></b> |
| <b>NSC</b>   | <b>HLA-G</b>  | 79.7                            | 97.1                            |
|              | <b>SOX2</b>   | 81.8                            | 97.5                            |
|              | <b>NESTIN</b> | 99.7                            | 99.6                            |
|              | <b>PAX6</b>   | 2.64                            | 0.27                            |
|              | <b>CDX2</b>   | 99.6                            | 97.7                            |

|  |             |      |      |
|--|-------------|------|------|
|  | <b>NCAD</b> | 11.5 | 5.87 |
|  | <b>CD90</b> | 99.8 | 99.8 |
|  | <b>CD44</b> | 99.7 | 99.8 |

| <b>MPSC1</b> | <b>Marker</b> | <b>STEMULATE<br/>Control</b> | <b>STEMULATE +<br/>RA</b> |
|--------------|---------------|------------------------------|---------------------------|
|              |               | <b>Mean FL + G2*</b>         | <b>Mean FL + G2*</b>      |
| <b>NSC</b>   | <b>HLA-G</b>  | 19.7                         | 27.6                      |
|              | <b>SOX2</b>   | 36.1                         | 37.9                      |
|              | <b>NESTIN</b> | 292                          | 181                       |
|              | <b>PAX6</b>   | 51.8                         | 178                       |
|              | <b>CDX2</b>   | 52.3                         | 33.1                      |
|              | <b>NCAD</b>   | 20.4                         | 40.2                      |
|              | <b>CD90</b>   | 815                          | 785                       |
|              | <b>CD44</b>   | 258                          | 252                       |

*MPSC1 Culture Condition 2: Neural Stem Cell Markers*

| <b>MPSC1</b> | <b>Marker</b> | <b>MESENCULT<br/>Control</b> | <b>MESENCULT RA</b>  |
|--------------|---------------|------------------------------|----------------------|
|              |               | <b>% + cells G2*</b>         | <b>% + cells G2*</b> |
| <b>NSC</b>   | <b>HLA-G</b>  | 79.3                         | 98.8                 |
|              | <b>SOX2</b>   | 39.2                         | 80.6                 |
|              | <b>NESTIN</b> | 99.9                         | 99.9                 |
|              | <b>PAX6</b>   | 0.27                         | 1.81                 |
|              | <b>CDX2</b>   | 99.8                         | 99.8                 |
|              | <b>NCAD</b>   | 40.2                         | 19.8                 |
|              | <b>CD90</b>   | 99.9                         | 99.7                 |
|              | <b>CD44</b>   | 99.9                         | 99.8                 |

| <b>MPSC1</b> | <b>Marker</b> | <b>MESENCULT</b> | <b>MESENCULT RA</b> |
|--------------|---------------|------------------|---------------------|
|--------------|---------------|------------------|---------------------|

|            |               | <b>Control</b>       |                      |
|------------|---------------|----------------------|----------------------|
|            |               | <b>Mean FL + G2*</b> | <b>Mean FL + G2*</b> |
| <b>NSC</b> | <b>HLA-G</b>  | 16.7                 | 29.9                 |
|            | <b>SOX2</b>   | 20.1                 | 24.3                 |
|            | <b>NESTIN</b> | 227                  | 265                  |
|            | <b>PAX6</b>   | 114                  | 47.1                 |
|            | <b>CDX2</b>   | 40                   | 46.3                 |
|            | <b>NCAD</b>   | 21.7                 | 18.7                 |
|            | <b>CD90</b>   | 781                  | 724                  |
|            | <b>CD44</b>   | 236                  | 245                  |

*MPSC2 Culture Condition 1: Neural Stem Cell Markers*

| <b>MPSC2</b> | <b>Marker</b> | <b>STEMULATE<br/>Control</b> | <b>STEMULATE RA</b> |
|--------------|---------------|------------------------------|---------------------|
|              |               | % + cells G2*                | % + cells G2*       |
| <b>NSC</b>   | <b>HLA-G</b>  | 98                           | 98.3                |
|              | <b>SOX2</b>   | 95.3                         | 36.5                |
|              | <b>NESTIN</b> | 99.6                         | 99.4                |
|              | <b>PAX6</b>   | 14.5                         | 2.58                |
|              | <b>CDX2</b>   | 99.4                         | 99.2                |
|              | <b>NCAD</b>   | 9.23                         | 9.4                 |
|              | <b>CD90</b>   | 99.8                         | 99.6                |
|              | <b>CD44</b>   | 99.6                         | 98.8                |

| <b>MPSC2</b> | <b>Marker</b> | <b>STEMULATE<br/>Control</b> | <b>STEMULATE RA</b> |
|--------------|---------------|------------------------------|---------------------|
|              |               | Mean FL + G2*                | Mean FL + G2*       |
| <b>NSC</b>   | <b>HLA-G</b>  | 67.4                         | 55.9                |
|              | <b>SOX2</b>   | 52.5                         | 34.7                |
|              | <b>NESTIN</b> | 527                          | 408                 |

|  |             |      |      |
|--|-------------|------|------|
|  | <b>PAX6</b> | 46   | 71.4 |
|  | <b>CDX2</b> | 88.7 | 73.9 |
|  | <b>NCAD</b> | 119  | 52.3 |
|  | <b>CD90</b> | 651  | 673  |
|  | <b>CD44</b> | 337  | 240  |

*MPSC2 Culture Condition 2: Neural Stem Cell Markers*

| <b>MPSC2</b> | <b>Marker</b> | <b>MESENCULT<br/>Control</b> | <b>MESENCULT RA</b> |
|--------------|---------------|------------------------------|---------------------|
|              |               | % + cells G2*                | % + cells G2*       |
| <b>NSC</b>   | <b>HLA-G</b>  | 99.4                         | 98.3                |
|              | <b>SOX2</b>   | 87.5                         | 23.4                |
|              | <b>NESTIN</b> | 99.7                         | 99.8                |
|              | <b>PAX6</b>   | 0.33                         | 0.68                |
|              | <b>CDX2</b>   | 99.6                         | 99                  |
|              | <b>NCAD</b>   | 18.5                         | 10.1                |
|              | <b>CD90</b>   | 99.8                         | 99.5                |
|              | <b>CD44</b>   | 96.7                         | 96.6                |

| <b>MPSC2</b> | <b>Marker</b> | <b>MESENCULT<br/>Control</b> | <b>MESENCULT RA</b> |
|--------------|---------------|------------------------------|---------------------|
|              |               | Mean FL + G2*                | Mean FL + G2*       |
| <b>NSC</b>   | <b>HLA-G</b>  | 120                          | 71.4                |
|              | <b>SOX2</b>   | 48.4                         | 31.3                |
|              | <b>NESTIN</b> | 1830                         | 736                 |
|              | <b>PAX6</b>   | 144                          | 65.4                |
|              | <b>CDX2</b>   | 98.4                         | 66.1                |
|              | <b>NCAD</b>   | 52.1                         | 71.5                |
|              | <b>CD90</b>   | 602                          | 422                 |
|              | <b>CD44</b>   | 200                          | 165                 |

[00174] NCS markers of the produced cells include one or more of: NCAD, NESTIN, SOX2, PAX6, or any combination thereof. In one instance, a NCS cell comprises one of NCAD, NESTIN, SOX2, and PAX6. In one instance, a NCS cell comprises two of NCAD, NESTIN, SOX2, and PAX6 (*e.g.*, NCAD and NESTIN, NCAD and SOX2, NCAD and PAX6, NESTIN and SOX2, NESTIN and PAX6, or SOX2 and PAX6). In one instance, a NCS cell comprises three of NCAD, NESTIN, SOX2, and PAX6 (*e.g.*, CAD/NESTIN/SOX2, CAD/NESTIN/PAX6, NESTIN/SOX2/PAX6). In one instance, a NCS cell comprises all of NCAD, NESTIN, SOX2, and PAX6.

*MPSC1 Culture Condition 1: Pancreatic Progenitor Cell Markers*

| MPSC1 | Marker | STEMULATE Control | STEMULATE FGF |
|-------|--------|-------------------|---------------|
|       |        | % + cells G2*     | % + cells G2* |
| PPC   | HLA-G  | 97.7              | 98.7          |
|       | CDX2   | 98.8              | 97            |
|       | FOXA2  | 26                | 50.8          |
|       | SOX9   | 11.8              | 38.9          |
|       | PDX1   | 9.41              | 30.9          |
|       |        | % - cells         | % - cells     |
|       | CD34   | 98.2              | 98.9          |
|       | CD45   | 98.1              | 98.4          |

| MPSC1 | Marker | STEMULATE Control | STEMULATE FGF |
|-------|--------|-------------------|---------------|
|       |        | Mean FL + G2*     | Mean FL + G2* |
| PPC   | HLA-G  | 27.3              | 30.5          |
|       | CDX2   | 53.2              | 41.4          |
|       | FOXA2  | 10.6              | 10.9          |
|       | SOX9   | 15.8              | 12.2          |
|       | PDX1   | 25                | 30.4          |
|       |        | Mean FL -         | Mean FL -     |
|       | CD34   | 9.65              | 8.67          |

|  |             |      |      |
|--|-------------|------|------|
|  | <b>CD45</b> | 9.52 | 8.82 |
|--|-------------|------|------|

*MPSC1 Culture Condition 2: Pancreatic Progenitor Cell Markers*

| MPSC1      | Marker       | MESENCULT™<br>Control | MESENCULT™<br>FGF |
|------------|--------------|-----------------------|-------------------|
|            |              | % + cells G2*         | % + cells G2*     |
| <b>PPC</b> | <b>HLA-G</b> | 98.2                  | 95.8              |
|            | <b>CDX2</b>  | 93.7                  | 99.6              |
|            | <b>FOXA2</b> | <b>55.5</b>           | <b>13.9</b>       |
|            | <b>SOX9</b>  | <b>20.7</b>           | <b>15.9</b>       |
|            | <b>PDX1</b>  | <b>47.3</b>           | <b>19.8</b>       |
|            |              | % - cells             | % - cells         |
|            | <b>CD34</b>  | 99.5                  | 99.5              |
|            | <b>CD45</b>  | 99.4                  | 99.5              |

[00175] Bold text indicates markers with consistently increased or decreased after FBF treatment.

| MPSC1      | Marker       | MESENCULT™<br>Control | MESENCULT™<br>FGF |
|------------|--------------|-----------------------|-------------------|
|            |              | Mean FL + G2*         | Mean FL + G2*     |
| <b>PPC</b> | <b>HLA-G</b> | 23.8                  | 21                |
|            | <b>CDX2</b>  | 33.9                  | 61.9              |
|            | <b>FOXA2</b> | 10.5                  | 10.2              |
|            | <b>SOX9</b>  | 11                    | 10.7              |
|            | <b>PDX1</b>  | 25.1                  | 21.5              |
|            |              | Mean FL -             | Mean FL -         |
|            | <b>CD34</b>  | 8.41                  | 8.52              |
|            | <b>CD45</b>  | 8.45                  | 8.55              |

*MPSC2 Culture Condition 1: Pancreatic Progenitor Cell Markers*

| MPSC2 | Marker | STEMULATE Control | STEMULATE FGF |
|-------|--------|-------------------|---------------|
|-------|--------|-------------------|---------------|

|            |              | % + cells G2* | % + cells G2* |
|------------|--------------|---------------|---------------|
| <b>PPC</b> | <b>HLA-G</b> | 99.6          | 99.3          |
|            | <b>CDX2</b>  | 98.5          | 98.4          |
|            | <b>FOXA2</b> | 34.7          | 41.2          |
|            | <b>SOX9</b>  | 52.1          | 69.3          |
|            | <b>PDX1</b>  | 43.1          | 24.2          |
|            |              | % - cells     | % - cells     |
|            | <b>CD34</b>  | 96.6          | 96.4          |
|            | <b>CD45</b>  | 96.5          | 96.7          |

| <b>MPSC2</b> | <b>Marker</b> | <b>STEMULATE Control</b> | <b>STEMULATE FGF</b> |
|--------------|---------------|--------------------------|----------------------|
|              |               | Mean FL + G2*            | Mean FL + G2*        |
| <b>PPC</b>   | <b>HLA-G</b>  | 106                      | 105                  |
|              | <b>CDX2</b>   | 95.5                     | 108                  |
|              | <b>FOXA2</b>  | 35.2                     | 35.5                 |
|              | <b>SOX9</b>   | 35.3                     | 35.7                 |
|              | <b>PDX1</b>   | 59.4                     | 52.2                 |
|              |               | Mean FL -                | Mean FL -            |
|              | <b>CD34</b>   | 19.4                     | 19.5                 |
|              | <b>CD45</b>   | 19.7                     | 18.8                 |

*MPSC2 Culture Condition 2: Pancreatic Progenitor Cell Markers*

| <b>MPSC2</b> | <b>Marker</b> | <b>MESENCULT™<br/>Control</b> | <b>MESENCULT™<br/>FGF</b> |
|--------------|---------------|-------------------------------|---------------------------|
|              |               | % + cells G2*                 | % + cells G2*             |
| <b>PPC</b>   | <b>HLA-G</b>  | 99.5                          | 99.6                      |
|              | <b>CDX2</b>   | 99.5                          | 98.7                      |
|              | <b>FOXA2</b>  | 28.1                          | 28.4                      |
|              | <b>SOX9</b>   | 56.2                          | 49.8                      |
|              | <b>PDX1</b>   | 13.1                          | 43.5                      |

|  |             |           |           |
|--|-------------|-----------|-----------|
|  |             | % - cells | % - cells |
|  | <b>CD34</b> | 98.9      | 99.1      |
|  | <b>CD45</b> | 99.2      | 99.2      |

| MPSC2      | Marker       | MESENCULT™<br>Control | MESENCULT™<br>FGF |
|------------|--------------|-----------------------|-------------------|
|            |              | Mean FL + G2*         | Mean FL + G2*     |
| <b>PPC</b> | <b>HLA-G</b> | 168                   | 201               |
|            | <b>CDX2</b>  | 145                   | 101               |
|            | <b>FOXA2</b> | 41.5                  | 34.5              |
|            | <b>SOX9</b>  | 41.5                  | 34.6              |
|            | <b>PDX1</b>  | 109                   | 67.4              |
|            |              | Mean FL -             | Mean FL -         |
|            | <b>CD34</b>  | 13.3                  | 12.3              |
|            | <b>CD45</b>  | 13.1                  | 11.9              |

[00176] PPC markers of the produced cells include one or more of: PDX1, FOXA2, SOC9, or any combination thereof. In one instance, a PPC cell comprises one of PDX1, FOXA2, and SOC. In another instance, a PPC cell comprises two of PDX1, FOXA2, and SOC. For example, a PPC can comprise PDX1 and FOXA2, PDX1 and SOC, or FOXA2 and SOC. In another instance, a PPC cell comprises all of PDX1, FOXA2, and SOC.

#### Example 14. MPSCs Manufacturing Scale Up

|                                                          | Growth Conditions                      |                                                                 |                                     |                                                                           |                                                    |
|----------------------------------------------------------|----------------------------------------|-----------------------------------------------------------------|-------------------------------------|---------------------------------------------------------------------------|----------------------------------------------------|
| MPSC1                                                    | Culture Format                         | Media and Microcarriers                                         | Supplements                         | Cell density                                                              | Assays                                             |
| <b>1. Evaluate microcarrier adherence and suspension</b> | A. Non-treated 6-well plates on shaker | MESENCULT™<br>- ACF.<br>Corning Low Concentration<br>SYNTHEMAX® | BSA, 0.5% +<br>Pluronic F68<br>0.1% | (1) 3,000 cells/cm <sup>2</sup><br>(2) 5,000 cells/cm <sup>2</sup><br>(3) | Cell count, viability at 3 days<br>expansion<br>no |

|                                                   |                                                 |                                                                                         |                               |                              |                                                                                                                               |
|---------------------------------------------------|-------------------------------------------------|-----------------------------------------------------------------------------------------|-------------------------------|------------------------------|-------------------------------------------------------------------------------------------------------------------------------|
| <b>expansion</b>                                  | (70 rpm)                                        | II Microcarriers                                                                        |                               | 10,000 cells/cm <sup>2</sup> | significant cell attachment to microcarriers was observed at 3 days expansion                                                 |
|                                                   | B. Non-treated 6-well plates on shaker (70 rpm) | MESENCULT <sup>TM</sup> - ACF.<br>Corning Low Concentration SYNTHEMAX® II Microcarriers | BSA, 0.5% + Pluronic F68 0.1% | 10,000 cells/cm <sup>2</sup> | Cell count, viability at 3 days expansion. Increased cell attachment to microcarriers and proliferation using Rooster- MSC-XF |
|                                                   |                                                 | Rooster-MSC-XF.<br>Corning Low Concentration SYNTHEMAX® II Microcarriers                | none                          |                              |                                                                                                                               |
| <b>2. Expansion at 100 mL scale in suspension</b> | 100 mL bioreactor (25-30 rpm)                   | Rooster-MSC-XF.<br>Corning Low Concentration SYNTHEMAX® II Microcarriers                | none                          | 4600 cells/cm <sup>2</sup>   | (1) Cell count, viability at 3 days and 7 days expansion<br>(2) FACS surface marker                                           |

|  |  |  |  |  |                                      |
|--|--|--|--|--|--------------------------------------|
|  |  |  |  |  | expression at<br>7 days<br>expansion |
|--|--|--|--|--|--------------------------------------|

[00177] MPSC1 cells (about 10000 cells/cm<sup>2</sup>) were cultured with microcarriers on a shaker. After a 3-day expansion in (1) MESENCULT™, (2) MESENCULT™ + BSA, (3) MESENCULT™ + PLU, or (4) Rooster media, cells were analyzed by Trypan-blue exclusion test and live/dead imaging.

[00178] FIG. 11A is a graph illustrating cell numbers at 72 hours. In each bar, dead cells are in the top, and live cells are below. FIG. 11B is a graph illustrating population doublings in each type of media.

#### Example 15. MPSC Expansion in a Bioreactor

[00179] MPSCs (about 4,600 cells/cm<sup>2</sup>) with microcarriers were cultured in Rooster MXC XF medium in a 100 mL PBS bioreactor at 25 rpm for about 7 days. Cell counts and viability were determined. FACS characterization of cell marks was conducted comparing adherence vs. suspension cultures.

[00180] FIG. 12A is a graph demonstrating cell counts on different days of culture.  $\Delta_{D2-D6}$  = 44,000,000 cells. FIG. 12B is a graph demonstrating % live cells during culture. FIG. 12C is a graph demonstrating population doublings comparing adherence vs. suspension cultures.  $\Delta_{D2-D6}$  = 4.9 PD. While adherent cultures initially doubled faster than suspended cultures, over time, suspended cultures achieved a higher rate of population doubling.

[00181] MPSC and NK markers for adherent and suspended cell cultures are as shown below.

| MSC MARKERS                               | MPSC1 Adherent | MPSC1 Suspension |
|-------------------------------------------|----------------|------------------|
| <b>NEGATIVE MARKERS: % Negative Cells</b> |                |                  |
| <b>CD4</b>                                | 98.5           | 99.2             |
| <b>CD14</b>                               | 98.9           | 99.3             |
| <b>CD19</b>                               | 98.9           | 99.3             |
| <b>CD34</b>                               | 98.9           | 99.4             |
| <b>CD45</b>                               | 99.0           | 99.3             |
| <b>HLA-DR</b>                             | 98.8           | 99.4             |
| <b>POSITIVE MARKERS: % Positive Cells</b> |                |                  |

|              |      |      |
|--------------|------|------|
| <b>CD29</b>  | 99.9 | 99.9 |
| <b>CD44</b>  | 98.7 | 98.0 |
| <b>CD73</b>  | 99.9 | 99.4 |
| <b>CD90</b>  | 99.7 | 97.3 |
| <b>CD105</b> | 98.2 | 93.6 |
| <b>CD146</b> | 90.4 | 35.8 |
| <b>CD166</b> | 99.8 | 99.9 |
| <b>HLA-I</b> | 99.9 | 99.9 |

| <b>MSC MARKERS</b>                                        | <b>MPSC1 Adherent</b> | <b>MPSC1 Suspension</b> |
|-----------------------------------------------------------|-----------------------|-------------------------|
| <b>NEGATIVE MARKERS: Mean Fluorescence Negative Cells</b> |                       |                         |
| <b>CD4</b>                                                | 6.9                   | 6.56                    |
| <b>CD14</b>                                               | 6.64                  | 6.67                    |
| <b>CD19</b>                                               | 6.57                  | 6.64                    |
| <b>CD34</b>                                               | 6.57                  | 6.71                    |
| <b>CD45</b>                                               | 6.22                  | 6.68                    |
| <b>HLA-DR</b>                                             | 6.42                  | 6.6                     |
| <b>POSITIVE MARKERS: Mean Fluorescence Positive Cells</b> |                       |                         |
| <b>CD29</b>                                               | 2021                  | 1700                    |
| <b>CD44</b>                                               | 145                   | 120                     |
| <b>CD73</b>                                               | 367                   | 257                     |
| <b>CD90</b>                                               | 335                   | 295                     |
| <b>CD105</b>                                              | 354                   | 176                     |
| <b>CD146</b>                                              | 187                   | 45.7                    |
| <b>CD166</b>                                              | 1772                  | 899                     |
| <b>HLA-I</b>                                              | 457                   | 482                     |

| <b>NK MARKERS</b>                         | <b>MPSC1 Adherent</b> | <b>MPSC1 Suspension</b> |
|-------------------------------------------|-----------------------|-------------------------|
| <b>POSITIVE MARKERS: % Positive Cells</b> |                       |                         |
| <b>IgG2B</b>                              | 0.5                   | 0.1                     |

|             |      |      |
|-------------|------|------|
| <b>CD56</b> | 12.5 | 1.34 |
|-------------|------|------|

| <b>NK MARKERS</b>                                         | <b>MPSC1 Adherent</b> | <b>MPSC1 Suspension</b> |
|-----------------------------------------------------------|-----------------------|-------------------------|
| <b>POSITIVE MARKERS: Mean Fluorescence Positive Cells</b> |                       |                         |
| <b>IgG2B</b>                                              | 28.3                  | 18.5                    |
| <b>CD56</b>                                               | 19.2                  | 23.2                    |

*Further optimization*

| <b>Parameters</b>                 | <b>Optimization</b>                                                                                                                                                                                                                                              |
|-----------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>1. Media and microcarriers</b> | <ul style="list-style-type: none"> <li>• MESENCULT™ medium + supplements</li> <li>• CORNING® Enhanced Attachment Microcarriers, 10g vial (Corning Cat# 3779)</li> <li>• CORNING® SYNTHEMAX® II Dissolvable Microcarriers, 1g vial (Corning Cat# 4989)</li> </ul> |
| <b>2. Seeding conditions</b>      | <ul style="list-style-type: none"> <li>• Cell density: 3,000-10,000 cells/cm<sup>2</sup></li> <li>• Incubation time cells + microcarriers before suspension initiation</li> <li>• Stirring speed bioreactor</li> </ul>                                           |
| <b>3. Subculture conditions</b>   | <ul style="list-style-type: none"> <li>• Enzymatic dissociation time</li> <li>• Subculture interval</li> </ul>                                                                                                                                                   |

*Summary of Findings*

**[00182]** Growth curve analyses show different growth properties between different MPSC lines: MPSC4 < MPSC3 < MPSC2 < MPSC1. FACS characterization for MSC/NK markers show heterogeneous cell population for the MPSC4 cell line; MPSC2, MPSC3, and MPSC1 cell lines are homogeneous and express core MSC markers. Short-term differentiation into PPC and NSC using MESENCULT™ and MEM-α+STEMULATE media shows media-dependent marker expression; both MPSC1 and MPSC2 cell lines express combinations of PPC, NSC, and MPSC markers following differentiation. In co-culture with activated T cell like lines (*e.g.*, Jurkat cells), MPSCs were able to stimulate activated IL-2 secretion in Jurkat cells.

**[00183]** While some embodiments have been shown and described herein, such embodiments are provided by way of example only. Numerous variations, changes, and substitutions can occur without departing from the inventions. It should be understood that various alternatives to the embodiments of the inventions described herein can be employed in practicing the inventions.