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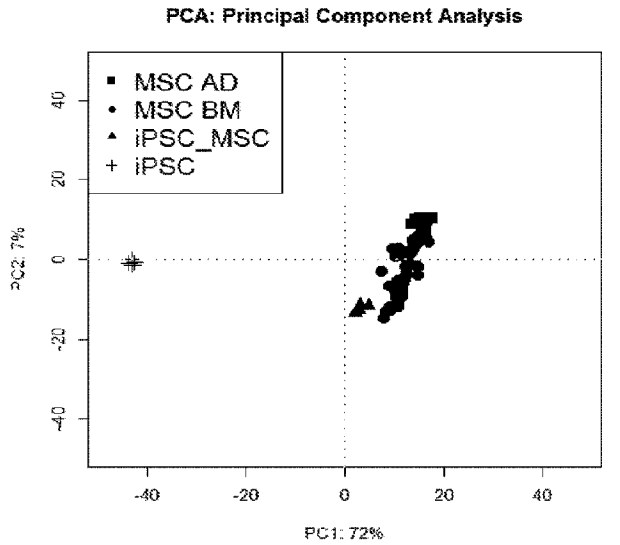

(57) Abrégé/Abstract:
The invention relates to a method for producing a mesenchymal stem cell (MSC), the method comprising culturing a primitive mesoderm cell in a mesenchymal colony forming medium (M-CFM) comprising LiCl and FGF2, but excluding PDGF, under normoxic conditions for sufficient time for a mesenchymal colony to form, and culturing the mesenchymal colony adherently to produce the MSC, wherein the MSC has superior T-cell immunosuppressive properties relative to an MSC not produced in said M-CFM. The invention also relates to an MSC produced by the method, a population of MSCs produced by the method, a therapeutic composition comprising the MSC produced by the method, an M-CFM and an M-CFM in concentrated form, and method and uses of the MSC or population in treating a disease.

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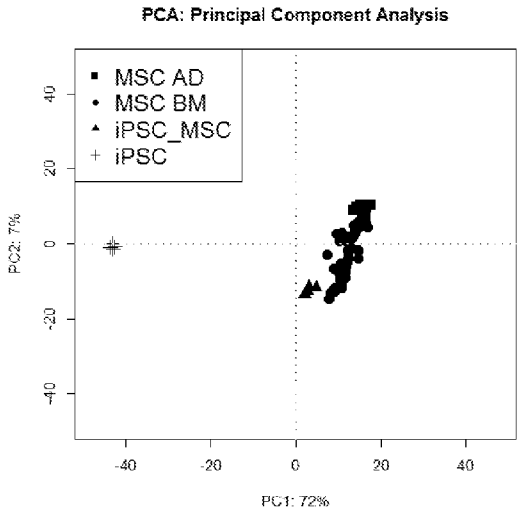

Figure 9.

(57) **Abstract**: The invention relates to a method for producing a mesenchymal stem cell (MSC), the method comprising culturing a primitive mesoderm cell in a mesenchymal colony forming medium (M-CFM) comprising LiCl and FGF2, but excluding PDGF, under normoxic conditions for sufficient time for a mesenchymal colony to form, and culturing the mesenchymal colony adherently to produce the MSC, wherein the MSC has superior T-cell immunosuppressive properties relative to an MSC not produced in said M-CFM. The invention also relates to an MSC produced by the method, a population of MSCs produced by the method, a therapeutic composition comprising the MSC produced by the method, an M-CFM and an M-CFM in concentrated form, and method and uses of the MSC or population in treating a disease.

WO 2017/156580 A1

COLONY FORMING MEDIUM AND USE THEREOF

FIELD

The invention relates to a method of differentiating a human pluripotent stem cell (PSC) into a mesenchymal stem cell (MSC), and to compositions useful for such differentiation. The invention also relates to a MSC differentiated by the method, a population of the MSCs differentiated by the method, and to methods and uses of the MSC or population of MSCs.

BACKGROUND

Pluripotent stem cells (PSCs), in particular human PSCs, which include Embryonic Stem Cells (ESCs) and induced Pluripotent Stem Cells (iPSCs), provide the opportunity to study human development *in vitro* and develop novel cell-based therapeutic products. The use of PSCs as a starting material for the manufacture of therapeutic products has several advantages, including the potential to manufacture a virtually limitless number of cells from a single cell bank, as a result of the capacity of PSCs to reproduce indefinitely *in vitro*, and to differentiate into any cell type.

Most protocols differentiate human PSCs grown on mouse embryonic fibroblasts (MEFs) or MATRIGEL®, a solubilised basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. A chemically-defined, xenogen-free medium and matrix for human PSC-derivation and maintenance has been described. A chemically-defined, xenogen-free directed differentiation protocol for deriving hematopoietic progenitors from human PSCs has also been described in US 2014/0273211 A1.

In particular, US 2014/0273211 A1 describes a method for differentiating human pluripotent stem cells comprising: (a) providing human pluripotent stem cells; and (b) culturing the human pluripotent stem cells under hypoxic conditions in a cell culture medium comprising FGF2, BMP4, Activin A, and LiCl for a period of about two days to form a cell population of ^{EMH}lin-KDR+APLNR+PDGFRalpha+ primitive mesoderm cells with mesenchymoangioblast potential. US 2014/0273211 A1 also describes a

xenogen-free culture medium for differentiating human pluripotent stem cells, comprising IF9S medium supplemented with: about 50 to about 250 ng/mL BMP4; about 10 to about 15 ng/mL Activin A; about 10 to about 50 ng/mL FGF2; and about 1 mM to about 2 mM LiCl.

However, US 2014/0273211 A1 is silent in respect of any improved mesenchymal-colony forming medium (M-CFM).

Mesenchymal stem cells (MSCs) hold significant promise as a cell therapy and are currently in clinical trials for treating numerous diseases, including graft-versus host disease. Induced pluripotent stem cell (iPSC)-derived MSCs have a unique advantage over directly sourced MSCs, i.e. derived from tissues such as bone marrow, umbilical cord blood, adipose tissue, because *in vitro* expansion of iPSCs can provide a virtually unlimited supply of MSCs.

Nevertheless, despite their promise, there remain challenges in producing MSCs *in vitro*, for example scaling up production from laboratory scale to good manufacturing practice (GMP) scale, which for any biological product is neither facile nor necessarily predictable, as will be appreciated in the art.

It follows that there is a need for improved and scalable methods for differentiating PSCs, including iPSCs, into MSCs.

If any publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common general knowledge in the art in Australia or any other country.

SUMMARY

The inventors have identified a specific composition for a differentiation medium that differentiates PSCs to MSCs, wherein such MSCs possess superior immunosuppressive properties relative to MSCs differentiated in the absence of the differentiation medium disclosed herein. Furthermore, the method is applicable to GMP-scale, i.e. a scale sufficient to supply MSCs for therapeutic use.

In a first aspect, the invention provides a MSC expressing the miR-145-5p, miR-181b-5p and miR-214-3p, but not miR-127-3p or miR-299-5p.

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In one embodiment, the MSC has a CD73+CD105+CD90+CD146+CD44+CD10+CD31-CD45- phenotype.

In a second aspect, the invention provides a method for producing an MSC, the method comprising:

(a) culturing a primitive mesoderm cell in a M-CFM comprising LiCl and FGF2, but excluding PDGF, under normoxic conditions for sufficient time for a mesenchymal colony to form; and

(b) culturing the mesenchymal colony of (a) adherently to produce the MSC,

wherein the MSC of (b) has superior T-cell immunosuppressive properties relative to an MSC not produced in said M-CFM.

In one embodiment, the primitive mesodermal cell is a primitive mesodermal cell with mesenchymoangioblast (MCA) potential. In one embodiment, the primitive mesodermal cell with MCA potential has a $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ phenotype.

In one embodiment, sufficient time for the mesenchymal colony to be produced is about 8 days to about 14 days. In one embodiment, sufficient time for the mesenchymal colony to be produced is about 12 days.

In one embodiment, T-cell immunosuppressive properties of the MSC are determined relative to an MSC produced by a method comprising:

(a') culturing a primitive mesodermal cell in a medium comprising FGF2 and optionally PDGF, but excluding LiCl, under normoxic conditions for sufficient time for a mesenchymal colony to form; and

(b') culturing the mesenchymal colony of (a') adherently to produce the MSC.

In one embodiment, T-cell immunosuppressive properties of the MSC are determined relative to an MSC produced from a primitive mesodermal cell differentiated from a PSC in a differentiation medium comprising FGF2, BMP4, Activin A, and LiCl under hypoxic conditions for about two days.

In a third aspect, the invention provides an MSC produced by the method of the second aspect.

In a fourth aspect, the invention provides a population of MSCs, comprising the MSC of the first or third aspect. The population of MSCs

In a fifth aspect, the invention provides a therapeutic composition comprising the MSC of the first or third aspect, or the population of MSCs of the fourth aspect.

In a sixth aspect, the invention provides a kit comprising a container containing the MSC of the first or third aspect, the population of MSCs of the fourth aspect, or the therapeutic composition of the fifth aspect.

In a seventh aspect, the invention provides a mesenchymal colony forming medium (M-CFM), comprising about 1 mM LiCl, and about 5 ng/mL to about 100 ng/mL FGF2, or about 10 ng/mL to about 50 ng/mL FGF2, about 10 ng/mL FGF2, or about 20 ng/mL FGF2, but excluding PDGF.

In an eighth aspect, the invention provides a composition comprising LiCl and FGF2, but excluding PDGF, that, when admixed with a liquid comprising water, produces a M-CFM comprising about 1 mM LiCl, and about 5 ng/mL to about 100 ng/mL FGF2, or about 10 ng/mL to about 50 ng/mL FGF2, about 10 ng/mL FGF2, or about 20 ng/mL FGF2, but excluding PDGF.

In a ninth aspect, the invention provides use of the MSC of the first or third aspect or the population of MSCs of the fourth aspect in the manufacture of a medicament for treating or preventing a condition such as bone cysts, bone neoplasms, fractures, cartilage defects, osteoarthritis, ligament injury, osteogenesis imperfecta, osteonecrosis, osteoporosis, aplastic anaemia, graft versus host disease (GvHD), myelodysplastic syndrome; Type 1 diabetes, Type 2 diabetes, autoimmune hepatitis, liver cirrhosis, liver failure, dilated cardiomyopathy, heart failure, myocardial infarction, myocardial ischemia, Crohn’s disease, ulcerative colitis, burns, epidermolysis bullosa, lupus erythematosus, rheumatoid arthritis, Sjogren’s disease, systemic sclerosis, bronchopulmonary dysplasia, chronic obstructive airways disease, emphysema, pulmonary fibrosis, amyotrophic lateral sclerosis (ALS), Alzheimer’s disease, brain injury, ataxia, degenerative disc disease, multiple system atrophy, multiple sclerosis, Parkinson’s disease, retinitis pigmentosa,

Romberg's disease, spinal cord injury, stroke, muscular dystrophy, limb ischaemia, kidney injury, lupus nephritis, endometriosis and complications of bone marrow or solid organ transplantation..

In a tenth aspect, the invention provides a method for treating or preventing a condition such as bone cysts, bone neoplasms, fractures, cartilage defects, osteoarthritis, ligament injury, osteogenesis imperfecta, osteonecrosis, osteoporosis, aplastic anaemia, GvHD, myelodysplastic syndrome; Type 1 diabetes, Type 2 diabetes, autoimmune hepatitis, liver cirrhosis, liver failure, dilated cardiomyopathy, heart failure, myocardial infarction, myocardial ischemia, Crohn's disease, ulcerative colitis, burns, epidermolysis bullosa, lupus erythematosus, rheumatoid arthritis, Sjogren's disease, systemic sclerosis, bronchopulmonary dysplasia, chronic obstructive airways disease, emphysema, pulmonary fibrosis, ALS, Alzheimer's disease, brain injury, ataxia, degenerative disc disease, multiple system atrophy, multiple sclerosis, Parkinson's disease, retinitis pigmentosa, Romberg's disease, spinal cord injury, stroke, muscular dystrophy, limb ischaemia, kidney injury, lupus nephritis, endometriosis and complications of bone marrow or solid organ transplantation, the method comprising administering to a subject the MSC of the first or third aspect, the population of MSCs of the fourth aspect, or the therapeutic composition of the fifth aspect.

Also disclosed is a method of differentiating a pluripotent stem cell (PSC) into a mesenchymal stem cell (MSC), the method comprising:

(a) culturing the PSC in a differentiation medium comprising FGF2, BMP4, Activin A, and LiCl under hypoxic conditions for about two days to form a primitive mesoderm cell;

(b) replacing the differentiation medium of (a) with a mesenchymal colony forming medium (M-CFM) comprising LiCl and FGF2, but excluding PDGF;

(c) culturing the primitive mesoderm cell of (b) in the M-CFM of (b) under normoxic conditions for sufficient time for a mesenchymal colony to form; and

(d) culturing the mesenchymal colony of (c) adherently to produce the MSC,

wherein the MSC has superior T-cell immunosuppressive properties relative to an MSC not produced in said M-CFM.

In one embodiment, the method above further comprises:

(e) expanding the MSC of (d).

In one embodiment, the primitive mesodermal cell is a primitive mesodermal cell with mesenchymoangioblast (MCA) potential. In one embodiment, the primitive mesodermal cell with MCA potential is a $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cell with MCA potential.

In one embodiment, sufficient time for the mesenchymal colony to be form is about 8 days to about 14 days. In one embodiment, sufficient time for the mesenchymal colony to form is about 12 days.

In one embodiment, T-cell immunosuppressive properties of the MSC are determined relative to an MSC produced from a primitive mesodermal cell cultured in a medium comprising FGF2 and optionally PDGF, but excluding LiCl.

In one embodiment, T-cell immunosuppressive properties of the MSC are determined relative to an MSC produced from a primitive mesodermal cell differentiated from a PSC in a differentiation medium comprising FGF2, BMP4, Activin A, and LiCl under hypoxic conditions for about two days.

BRIEF DESCRIPTION OF THE FIGURES

Embodiments of the invention are described below by way of example only with reference to the accompanying figures in which

Figure 1 is an amino acid sequence (SEQ ID NO: 1) representing a polypeptide that as a homodimer is one example of human activin A.

Figure 2 is an amino acid sequence (SEQ ID NO: 2) representing a polypeptide that as a homodimer is one example of human BMP4. SEQ ID NO: 2 corresponds to amino acids 303 to 408 of an amino acid sequence of a full length BMP4 precursor.

Figure 3 is an amino acid sequence (SEQ ID NO: 3) representing a polypeptide that is one example of a human type I collagen alpha1 chain. Human type I collagen (collagen I) is a triple helix trimer that in one example has two alpha1 chains represented by SEQ ID NO: 3 and one alpha2 chain. SEQ ID NO: 3

corresponds to amino acids 162 to 1218 of a full length type I collagen alpha1 chain precursor.

Figure 4 is an amino acid sequence (SEQ ID NO: 4) representing a polypeptide that is one example of a human type IV collagen alpha1 chain. Human type IV collagen (collagen IV) is a triple helix heterotrimer that in one example comprises one alpha1 chain represented by SEQ ID NO: 4. SEQ ID NO: 4 corresponds to amino acids 173 to 1669 of a full length type IV collagen alpha1 chain precursor.

Figure 5 is an amino acid sequence (SEQ ID NO: 5) representing a polypeptide that is one example of human FGF2.

Figure 6 is an amino acid sequence (SEQ ID NO: 6) representing a polypeptide that as a dimer is one example of human fibronectin. SEQ ID NO: 6 corresponds to amino acids 32 to 2446 of a full length fibronectin precursor.

Figure 7 is an amino acid sequence (SEQ ID NO: 7) representing a polypeptide that as a homodimer is one example of human PDGF (PDGF-BB). SEQ ID NO: 7 corresponds to amino acids 82 to 190 of a full length PDGF subunit B precursor.

Figure 8 is an amino acid sequence (SEQ ID NO: 8) representing a polypeptide that as a hexamer is one example of human tenascin C. SEQ ID NO: 8 corresponds to amino acids 23 to 2201 of a full length tenascin C precursor.

Figure 9 is a plot representing a principal component analysis of miRNA expression of an MSC of the disclosure and 71 other MSC samples obtained by means other than the present method. AD, adipose (■); BM, bone marrow (♦); iPSC_MSC, MSC of the disclosure (▲); iPSC, iPSC before differentiation to an MSC of the disclosure (+).

Figure 10 is a schematic representation of the study of example 6.

Figure 11 is a schematic representation of the histology of the study of example 6.

Figure 12 comprises fluorescent photomicrographs of engraftment (human nuclei staining) of MSCs in the hearts of example 6. Human nuclei staining was performed at all levels in mid LV (cryo sections). There was no evidence of cell engraftment at

day 28. Human mitochondria staining was performed at basal LV (paraffin sections). There was no evidence of cell engraftment at day 28.

Figure 13 shows functional assessment (TTE) and comprises short axis of M-mode images of hearts of vehicle, BM MSC, and PSC-MSC recipients of example 6 at baseline and 1 month time points. The images show reduced ventricular dilatation and increased ventricular contractility in the PSC-MSC recipients compared to the vehicle and BM MSC recipients.

Figure 14 shows functional assessment (TTE) and comprises line graphs showing individual assessments and column graphs showing group assessment of fractional shortening (FS) of the infarcted hearts of example 6. In the vehicle group, baseline FS was higher in 1 rat injected with vehicle, but overall, no or minimal change in FS on day 28 compared to day 0 except for 1 rat (mean increase 1.39%). In the BM MSC group, 3/3 rats had worsening FS on day 28 compared to day 0 (mean decrease 2.3%). In the PSC-MSC group, 4/4 rats had increase in FS on day 28 compared to day 0 (mean increase 5.48%).

Figure 15 comprises photomicrographs showing scar size using picosirius red staining in infarcted hearts of example 6. The images show an obvious reduction in scar size using Picosirius Red Staining in the PSC-MSC treated groups compared to the vehicle and BM-MSC treated groups.

Figure 16 comprises fluorescent photomicrographs assessing angiogenesis (vWF staining) in the hearts of example 6. The images show no difference in size and number of vessels between groups.

Figure 17 is a schematic representation of a study similar to example 6.

Figure 18 is a scatter plot of percent weight change at day +19 of mice of example 7 relating to GvHD.

Figure 19 is a line graph showing GvHD disease severity in mice of example 7.

Figure 20 is a Kaplan-Meier plot showing GvHD survival of mice of example 7.

DETAILED DESCRIPTION

The inventors have defined an improved chemically-defined, xenogen-free differentiation protocol, including in particular, an improved chemically-defined, xenogen-free mesenchymal colony forming protocol. Surprisingly, the improved protocols provide MSCs with superior immunosuppressive properties when compared to existing differentiation and mesenchymal colony forming protocols.

In developing the improved chemically-defined, xenogen-free differentiation protocol, including the improved mesenchymal colony forming protocol, the inventors investigated several variables, including: presence/ absence of PDGF; presence/ absence of LiCl; low/ high activin A concentration; and low/ high seed density.

PDGF was tested because it was asserted in US 7,615,374 that "PDGF-BB improved growth of mesenchymal cells, but was not essential for colony formation". Surprisingly, however, the inventors describe herein that contrary to this prior teaching, absence of PDGF generated MSCs with superior immunosuppressive properties relative to MSCs generated by differentiation in medium comprising PDGF.

With respect to activin A, the inventors surprisingly found that immunosuppression was improved in MSCs differentiated in medium comprising the lower concentration of activin A relative to the higher concentration of activin A.

LiCl activates Wnt signaling and is included in differentiation medium to improve mesoderm induction during the first 48 hours of differentiation, which is understood in the art.

However, before the present invention, LiCl had not been used in a M-CFM to improve colony formation in clonogenic cultures. Again, this was a surprising advantage of the method disclosed herein.

Accordingly, disclosed herein are methods for differentiating PSCs (e.g. human ESCs or human iPSCs) under defined conditions. Such differentiation provides an MSC or MSC populations that may be a source for functional studies of these lineages as well as a source for clinical therapies.

The ability of MSCs to exert immunomodulatory/ immunosuppressive effects, in particular by suppressing T cells, is believed to be central to the therapeutic effects of MSCs in a wide

range of conditions, including GvHD, immune disorders including autoimmune disorders, cardiovascular disorders, orthopaedic disorders and rejection of transplanted solid organs. Some specific examples include bone cysts, bone neoplasms, fractures, cartilage defects, osteoarthritis, ligament injury, osteogenesis imperfecta, osteonecrosis, osteoporosis, aplastic anaemia, myelodysplastic syndrome; Type 1 diabetes, Type 2 diabetes, autoimmune hepatitis, liver cirrhosis, liver failure, dilated cardiomyopathy, heart failure, myocardial infarction, myocardial ischemia, Crohn's disease, ulcerative colitis, burns, epidermolysis bullosa, lupus erythematosus, rheumatoid arthritis, Sjogren's disease, systemic sclerosis, bronchopulmonary dysplasia, chronic obstructive airways disease, emphysema, pulmonary fibrosis, ALS, Alzheimer's disease, brain injury, ataxia, degenerative disc disease, multiple system atrophy, multiple sclerosis, Parkinson's disease, retinitis pigmentosa, Romberg's disease, spinal cord injury, stroke, muscular dystrophy, limb ischaemia, kidney injury, lupus nephritis, endometriosis and complications of bone marrow or solid organ transplantation,

MSCs are thought to perform a critical role in injury healing, and have been shown to be effective in treating tissue injury and degenerative diseases, including in the digestive system, for example in liver cirrhosis and liver failure, in the musculoskeletal system, in periodontal tissue, in diabetic critical limb ischemia, in osteonecrosis, in burn-related disorders, in myocardial infarction, in cornea damage, in the brain, in the spinal cord, in the lungs, and in treating radiation exposure.

MSCs have shown therapeutic outcomes in immune disorders, including graft-versus-host disease, systemic lupus erythematosus (SLE), Crohn's disease, multiple system atrophy, multiple sclerosis, amyotrophic lateral sclerosis, and stroke.

MSCs have been shown to exert immunosuppressive activities against T cells, B cells, dendritic cells, macrophages, and natural killer cells. While not wishing to be bound by theory, the underlying mechanisms may comprise immunosuppressive mediators, for example nitric oxide, indoleamine 2,3, dioxygenase, prostaglandin E2, tumour necrosis factor-inducible gene 6 protein, CCL-2, and

programmed death ligand 1. These mediators are expressed at a low level until stimulated, for example by an inflammatory cytokines, such as IFNγ, TNFα, and IL-17.

In some embodiments, MSCs of the disclosure may be engrafted into damaged tissues following administration and migration.

MSCs may be administered systemically or peripherally, for example by routes including intravenous (IV), intra-arterial, intramuscular, intraperitoneal, intracerobrospinal, subcutaneous (SC), intra-articular, intrasynovial, intrathecal, intracoronary, transendocardial, surgical implantation, topical and inhalation (e.g. intrapulmonary). MSCs may be administered in combination with a scaffold of biocompatible material.

MSCs may be administered before, during or after injury, disorder or disease progression. In one embodiment, MSCs are administered during inflammation. MSCs may be administered (a) as a preventative measure, (b) as soon as the relevant condition has been diagnosed, (c) when other treatments fail, and/or (d) when a condition advances to a pre-defined degree of severity.

In one embodiment, MSCs are pre-treated prior to administration. Pre-treatment may be with a growth factor or by gene editing, for example, where a growth factor may prime the MSC and gene editing may confer a new therapeutic property on the MSC.

Unless defined otherwise in this specification, technical and scientific terms used herein have the same meaning as commonly understood by the person skilled in the art to which this invention belongs and by reference to published texts.

It is to be noted that the term "a" or "an" refers to one or more, for example, "a molecule," is understood to represent one or more molecules. As such, the terms "a" or "an", "one or more," and "at least one" may be used interchangeably herein.

In the claims which follow and in the description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

The term "about" as used herein contemplates a range of values for a given number of ±25% the magnitude of that number. In other embodiments, the term "about" contemplates a range of values for a given number of ±20%, ±15%, ±10%, or ±5% the magnitude of that number. For example, in one embodiment, "about 3 grams" indicates a value of 2.7 to 3.3 grams (i.e. 3 grams ±10%), and the like.

Similarly, while differentiation processes include ordered, sequential events, the timing of the events may be varied by at least 25%. For example, while a particular step may be disclosed in one embodiment as lasting one day, the event may last for more or less than one day. For example, "one day" may include a period of about 18 to about 30 hours. In other embodiments, periods of time may vary by ±20%, ±15%, ±10%, or ±5% of that period of time. Periods of time indicated that are multiple day periods may be multiples of "one day," such as, for example, two days may span a period of about 36 to about 60 hours, and the like. In another embodiment, time variation may be lessened, for example, where day 2 is 48±3 hours from day 0; day 4 is 96±3 hours from day 0, and day 5 is 120 hours±3 hours from day 0.

As used herein, "pluripotent stem cell" or "PSC" refers to a cell that has the ability to reproduce itself indefinitely, and to differentiate into any other cell type. There are two main types of pluripotent stem cell: embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs).

As used herein, "embryonic stem cell" or "ESC" refers to a cell isolated from a five to seven day-old embryo donated with consent by patients who have completed *in vitro* fertilisation therapy, and have surplus embryos. The use of ESCs has been hindered to some extent by ethical concerns about the extraction of cells from human embryos.

Suitable human PSCs include H1 and H9 human embryonic stem cells.

As used herein, "induced pluripotent stem cell" or "iPSC" refers to an ESC-like cell derived from adult cells. iPSCs have very similar characteristics to ESCs, but avoid the ethical concerns associated with ESCs, since iPSCs are not derived from embryos.

Instead, iPSCs are typically derived from fully differentiated adult cells that have been "reprogrammed" back into a pluripotent state.

Suitable human iPSCs include, but are not limited to, iPSC 19-9-7T, MIRJT6i-mND1-4 and MIRJT7i-mND2-0 derived from fibroblasts and iPSC BM119-9 derived from bone marrow mononuclear cells. Other suitable iPSCs may be obtained from Cellular Dynamics International (CDI; Nasdaq: ICEL) of Madison, WI, USA.

In some embodiments, the PSCs are plated at an initial density of about 5000 cells/cm^2 to about 15,000 cells/cm^2, e.g., 6000 cells/cm^2, 7000 cells/cm^2, 8000 cells/cm^2, 9000 cells/cm^2, 10,000 cells/cm^2, 11,000 cells/cm^2, 12,000 cells/cm^2, 13,000 cells/cm^2, or 14,000 cells/cm^2.

In one embodiment, sufficient time for the mesenchymal colony to be produced is about 8 days to about 14 days. In one embodiment, sufficient time for the mesenchymal colony to be produced is about 10 days to about 14 days. In one embodiment, sufficient time for the mesenchymal colony to be produced is about 11 days to about 13 days. In one embodiment, sufficient time for the mesenchymal colony to be produced is about 8 days, about 9 days, about 10 days, about 11 days, about 13 days, or about 14 days. In one embodiment, sufficient time for the mesenchymal colony to be produced is about 12 days.

As used herein, "$^{EMH}lin^-KDR^+APLNR^+PDGFRalpha^+$ primitive mesoderm cell with mesenchymoangioblast (MCA) potential" refers to a cell expressing typical primitive streak and lateral plate/extraembryonic mesoderm genes. These cells have potential to form mesenchymoangioblast (MCA) and hemangioblast colonies in serum-free medium in response to FGF2. According to the method of present invention, these cells become mesenchymal stem cells (MSCs).

The term $^{EMH}lin^-$ denotes lack of expression of CD31, VE-cadherin endothelial markers, CD73 and Cd105 mesenchymal/endothelial markers, and CD43 and CD45 hematopoietic markers.

As used herein, "mesenchyme" or "mesenchymal" refers to to embryonic connective tissue that is derived from the mesoderm and that differentiates into hematopoietic tissue (lymphatic and circulatory systems) and connective tissue, such as bone and cartilage. However, MSCs do not differentiate into hematopoietic cells.

As used herein, "mesenchymal colony" refers to CD73- mesenchymal progenitors that precede true MSCs. Additional adherent culture of mesenchymal colonies, e.g. on fibronectin/collagen-coated plates, is required to produce CD73+ MSCs.

As used herein, "mesenchymal stem cell" or "MSC" refers to a particular type of stem cell that may be isolated from a wide range of tissues, including bone marrow, adipose tissue (fat), placenta and umbilical cord blood. MSCs are also known as "mesenchymal stromal cells". According to the method of present invention, MSCs are formed from $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cells with mesenchymoangioblast (MCA) potential.

MSCs secrete bioactive molecules such as cytokines, chemokines and growth factors and have the ability to modulate the immune system. MSCs have been shown to facilitate regeneration and effects on the immune system without relying upon engraftment. In other words, the MSCs themselves do not necessarily become incorporated into the host - rather, they exert their effects and are then eliminated within a short period of time. However, MSCs may be engrafted.

Therapeutic MSCs can be either "autologous" or "allogeneic". As used herein, "autologous" means a patient is treated with their own cells isolated from bone marrow or adipose tissue, for example, whereas "allogeneic" means that cells from a donor are used to treat other people. According to the present disclosure, allogeneic MSCs are derived from a donor via an iPSC.

Allogeneic MSCs have not been shown to cause immune reactions in other people, so they do not require immune-matching the donor to the recipient. This has important commercial advantages.

As used herein, "mesenchymoangioblast" or "MCA" refers to a cell that is a precursor to a MSC. A MSC may be produced by the method of the invention.

As used herein, "differentiating" refers to a process of a cell changing from one cell type to another, in particular a less specialized type of cell becoming a more specialized type of cell.

As used herein, "medium" or its plural "media" refers to a liquid or gel designed to support the growth of cells. In some embodiments, the cell culture medium comprises an IF9S medium. In

some embodiments, the medium employs a 9S concentrated medium supplement, wherein dilution of the 9S concentrated medium supplement in an IMDM/F12 base medium yields an IF9S cell culture medium. The concentrated 9S medium supplement comprises 9 supplements as follows: L-ascorbic acid 2-phosphate Mg^{2+} salt, 1-thioglycerol (monothioglycerol), additional sodium selenite, polyvinyl alcohol, GLUTAMAX™ (or glutamine), non-essential amino acids, chemically defined lipid concentrate, holo-transferrin, and insulin. In some embodiments, the concentrated 9S medium supplement comprises each component at a concentration 10× to 1000× of the final working concentration once diluted in a base medium. In one embodiment, an IF9S medium comprises IMDM, F12 and 9S as follows: IMDM 0.5x, F12 0.5x, sodium bicarbonate 2.1 mg/mL, L-ascorbic acid 2-phosphate Mg^{2+} salt (64 µg/mL), 1-thioglycerol(50 µg/mL (460 µM, 40 µL/L)), sodium selenite (in addition to any present in the base medium; 8.4 ng/mL), polyvinyl alcohol (10 mg/mL), GLUTAMAX™ (1×), non-essential amino acids (1×), chemically defined lipid concentrate (1×), Holo-Transferrin (10.6 µg/mL), and insulin (20µg/mL).

Although the presently disclosed media may include specific components (e.g. morphogens, small molecules, and hematopoietic cytokines), it is contemplated that other components with the same, equivalent, or similar properties may be used in addition to or in place of those disclosed, as are known in the art.

In some embodiments, components of an IF9S medium may be substituted. For example, ascorbic acid and 1-thioglycerol can be replaced with an optional supplement of a compound and/or a thiol-containing compound with antioxidant properties. GLUTAMAX™ can be replaced with an optional supplement of L-glutamine. "Non-essential amino acids", which is a general term for amino acids that the human body can produce from other amino acids, can be replaced with an optional supplement of amino acids. "Chemically defined lipid concentrate," which is a solution specifically distributed by Life Technologies, can be replaced with an optional supplement of lipids. Additional selenite, insulin, and holo-transferrin can be replaced with any insulin-transferrin-selenite supplement. Polyvinyl alcohol can be replaced with an optional supplement of a biologically inactive media thickening compound.

As used herein, "differentiation medium" refers to a medium designed to support the differentiation of cells, that is, supporting the process of a cell changing from one cell type to another. According to the method of the present invention, a differentiation medium is used to support the process of changing a human PSC into a $^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cell with mesenchymoangioblast (MCA) potential.

In some embodiments, the concentration, in the differentiation medium, of: BMP4 is about 10 ng/mL to about 250 mg/mL; activin A is about 1 ng/mL to about 15 ng/mL; FGF2 is about 5 ng/mL to about 50 ng/mL; and LiCl is about 1 mM to about 2 mM.

In some embodiments, the concentration of BMP4 is in the differentiation medium is about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 45 ng/mL, about 50 ng/mL, about 60 ng/mL, about 70 ng/mL, about 80 ng/mL, about 90 ng/mL, about 100 ng/mL, about 110 ng/mL, about 120 ng/mL, about 130 ng/mL, about 140 ng/mL, about 150 ng/mL, about 160 ng/mL, about 170 ng/mL, about 180 ng/mL, about 190 ng/mL, about 200 ng/mL, about 210 ng/mL, about 220 ng/mL, about 230 ng/mL, about 240 ng/mL, or about 250 ng/mL.

The concentration of activin A in the differentiation medium may be about 1 ng/mL, about 2 ng/mL, about 3 ng/mL, about 4 ng/mL, about 5 ng/mL, about 6 ng/mL, about 7 ng/mL, about 8 ng/mL, about 9 ng/mL, about 10 ng/mL, about 11 ng/mL, about 12 ng/mL, about 13 ng/mL, about 14 ng/mL, or about 15 ng/mL.

The concentration of FGF2 in the differentiation medium may be about 1 ng/mL, about 5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 45 ng/mL, or about 50 ng/mL.

The concentration of LiCl in the differentiation medium may be about 0.5 mM, about 0.6 mM, about 0.7 mM, about 0.8 mM, about 0.9 mM, about 1 mM, about 1.1mM, about 1.2 mM, about 1.3 mM, about 1.4 mM, about 1.5 mM, about 1.6 mM, about 1.7 mM, about 1.8 mM, about 1.9 mM, about 2 mM, about 2.1 mM, about 2.2 mM, about 2.3 mM, about 2.4 mM, or about 2.5 mM. Preferably, as understood in the art,

concentration of LiCl in the differentiation medium may be about 2 mM.

In some embodiments, the differentiation medium comprises a base medium, L-ascorbic acid 2-phosphate Mg^{2+} salt, 1-thioglycerol, sodium selenite (in addition to any present in the base medium), polyvinyl alcohol, GLUTAMAX™, non-essential amino acids, chemically defined lipid concentrate, holo-transferrin, and insulin. Suitable base media for the differentiation media described herein include, but are not limited to, Iscove's Modified Dulbecco's Medium/F12 (IMDM/F12), TeSR1 base medium (mTeSR1™ base medium, Stem Cell Technologies) without FGF2 and TGF-beta; DF4S base medium, which is Essential 8™ medium (Life Technologies; also known as "E8" medium) without FGF2 and TGF-beta, I4S base medium, which is DF4S base with Iscove's modified Dulbecco's medium (IMDM) instead of DMEM/F12, and IF4S base is DF4S base with IMDM/F12 instead of DMEM/F12. Preferably, the base medium to be used is albumin-free. IMDM/F12 is a highly enriched synthetic medium suited for rapidly proliferating, high-density cell cultures with an added nutrient mixture. These media are known to the person skilled in the art.

In some embodiments, the medium referred to herein as "IF9S", comprises IMDM/F12, L-ascorbic acid 2-phosphate Mg^{2+} salt, 1-thioglycerol, sodium selenite (in addition to any present in the base medium), polyvinyl alcohol, GLUTAMAX™, non-essential amino acids, chemically defined lipid concentrate, Holo-Transferrin, and insulin.

As used herein, "mesenchymal colony forming medium (M-CFM)" refers to a medium designed to support the formation of mesenchymal colonies from $^{EMM}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+}$ primitive mesoderm cells with mesenchymoangioblast (MCA) potential.

In some embodiments, the concentration, in the the M-CFM, of: LiCl is about 1 mM and FGF2 is about 10 ng/mL; or LiCl is about 1 mM and FGF2 is about 20 ng/mL.

The concentration of FGF2 in the M-CFM may be about 1 ng/mL, about 5 ng/mL, about 10 ng/mL, about 15 ng/mL, about 20 ng/mL, about 25 ng/mL, about 30 ng/mL, about 35 ng/mL, about 40 ng/mL, about 45 ng/mL, or about 50 ng/mL.

The concentration of LiCl in the M-CFM may be about 0.5 mM, about 0.6 mM, about 0.7 mM, about 0.8 mM, about 0.9 mM, about 1 mM, about 1.1mM, about 1.2 mM, about 1.3 mM, about 1.4 mM, about 1.5 mM, about 1.6 mM, about 1.7 mM, about 1.8 mM, about 1.9 mM, about 2 mM, about 2.1 mM, about 2.2 mM, about 2.3 mM, about 2.4 mM, or about 2.5 mM. Preferably, the concentration of LiCl in the M-CFM is 1 mM. As noted above, inclusion of LiCl in a M-CFM to improve colony formation in clonogenic cultures was not known prior to the present invention and provides clear advantages for the method disclosed herein over methods known in the art.

As referred to herein, the term "defined medium" means that the identity and quantity of each component of a medium is known.

In some embodiments, media disclosed herein comprise xenogenic materials. As used herein, "xenogen" or "xenogenic" refers to non-human, biologically derived materials. Nonetheless, xenogenic materials may be defined. For example, a xenogenic material may be a recombinant protein of xenogenic origin. In one embodiment, the differentiation medium and/or the M-CFM comprises one or more xenogenic (e.g., recombinant non-human proteins).

In some embodiments, the medium is or the media are xenogen-free. Of central importance for clinical therapies is the absence of xenogenic materials in the derived cell populations, i.e., no non-human cells, cell fragments, sera, proteins, and the like. In one embodiment, xenogen-free differentiated cells are obtained using tenascin C or collagen IV, which essentially replaces contact with OP9 cells used in earlier differentiation systems.

Advantageously, defined media may be xenogen-free, and incorporate human proteins isolated from natural sources, such as from placenta or other human tissues, or that can be produced using recombinant technology. In some embodiments, all proteins described herein are human. In some embodiments, all of the proteins used in the differentiation medium are human proteins. In some embodiments all of the proteins used in the M-CFM medium are human proteins. In some embodiments, all proteins described herein are human recombinant proteins. In some embodiments, all of the proteins used in the differentiation medium are recombinant human proteins. In

some embodiments all of the proteins used in the M-CFM medium are recombinant human proteins.

All proteins described herein are known to the person skilled in the art, and most if not all proteins described herein are available commercially.

Media disclosed herein may be also made in concentrated, including dried, forms that are diluted prior to use, such as 2×, 10×, 100×, or 1000× concentrations.

As used herein, "culturing" refers to the process by which cells are grown under controlled conditions.

Cells cannot be held in culture indefinitely owing to the increasing concentration of toxic metabolites, decreasing concentration of nutrients, and, for dividing cells, an increasing number of cells. As used herein, "passaging" refers to the process of producing a new culture with refreshed concentrations of nutrients, no toxic metabolites, and optionally a lower density of cells than the originating culture.

In one embodiment, passaging comprises: culturing the mesenchymal colony for about 3 days; culturing the mesenchymal colony on fibronectin and/or collagen I; and/or 1, 2, 3, 4, 5, or 6 passages. The number of passages may be 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25. Preferably the number of passages is 10 or fewer. More preferably, the number of passages is 5 or 6.

As used herein, "hypoxic" refers to conditions in which the oxygen concentration of the gas mixture is about 3% O_2 to about 10% O_2. In some embodiments, hypoxic conditions are about 5% O_2, but may be about 4% O_2, about 6% O_2, about 7% O_2, about 8% O_2, or about 9% O_2. In embodiments where a cell culture medium is allowed to equilibrate under hypoxic conditions, the cell culture medium becomes hypoxic owing to the lower concentration of oxygen dissolved in the medium compared to a cell culture medium equilibrated under normoxic conditions.

As used herein, "normoxic" refers to conditions in which the oxygen concentration of the gas mixture is about 20% O_2, but may be about 18% O_2, about 19% O_2, about 21% O_2, or about 22% O_2.

As used herein "AA" refers to activin A. In one embodiment, activin A exists as a homodimer of the polypeptide represented by the amino acid sequence provided in Figure 1 as SEQ ID NO: 1.

As used herein, "BMP4" refers to bone morphogenic protein 4. In one embodiment, BMP4 exists as a homodimer of the polypeptide represented by the amino acid sequence provided in Figure 2 as SEQ ID NO: 2.

In one embodiment, "type I collagen" or "collagen I" exists as a triple helix trimer comprising two of the polypeptide represented by the amino acid sequence provided in Figure 3 as SEQ ID NO: 3, and a third collagen I chain.

In one embodiment, "type IV collagen" or "collagen IV" exists as a triple helix heterotrimer comprising one of the polypeptide represented as the amino acid sequence provided in Figure 4 as SEQ ID NO: 4, and two additional collagen IV chains.

As used herein, "FGF2" refers to fibroblast growth factor 2, also known as basic fibroblast growth factor. In one embodiment, FGF2 exists as a polypeptide represented as the amino acid sequence provided in Figure 5 as SEQ ID NO: 5.

In one embodiment, fibronectin exists as a dimer of the polypeptide represented by the amino acid sequence provided in Figure 6 as SEQ ID NO: 6.

As used herein, "PDGF" refers to platelet derived growth factor. In one embodiment, PDGF exists as a homodimer of the B subunit polypeptide represented by the amino acid sequence provided in Figure 7 as SEQ ID NO: 7 (PDGF-BB). It is disclosed herein that supplementing M-CFM with PDGF (10 ng/mL) during culture had a significant negative impact on immune-suppression of iPSC-derived MSCs (4-fold reduction).

In one embodiment, "tenascin C" exists as a hexamer of the polypeptide represented by the amino acid sequence provided in Figure 8 as SEQ ID NO: 8.

In some embodiments, adherent human PSCs are cultured on tenascin C or provided on a substrate treated with tenascin C. In some embodiments, any of the above-referenced cells (e.g., human PSCs, human iPSCs) are cultured on tenascin C. In some embodiments, any of the described cells are seeded on a substrate treated with an

amount of tenascin C sufficient to adhere 10,000 cells/cm^2 to the substrate. In some embodiments, the tenascin C is human tenascin C (e.g. comprising an amino acid sequence represented by SEQ ID NO: 8. or provided by GenBank Accession No. CAA55309.1, or available commercially, e.g., Millipore Cat. No. CC065). In some embodiments, the substrate is treated with tenascin C at a concentration of at least about 0.25 µg/cm^2 to about 1 µg/cm^2, e.g., 0.3 µg/cm^2, 0.4 µg/cm^2, 0.5 µg/cm^2, 0.6 µg/cm^2, 0.7 µg/cm^2, 0.8 µg/cm^2, or 0.9 µg/cm^2. Cells can be grown on, e.g., tenascin C-coated cell culture dishes, multi-well cell culture plates, or microcarrier beads.

The use of tenascin C and hypoxic conditions may enable generation of $^{EMH}lin^-KDR^+APLNR^+PDGFRalpha^+$ primitive mesoderm cells with mesenchymoangioblast (MCA) potential and mesenchymal stem cells at higher percentages than compared to cells cultured on collagen IV or OP9 cells, such as greater than 10%, or greater than about 20%, or greater than about 30%, or greater than about 35%, or greater than about 40%, or greater than about 50%, or greater than about 60%, or greater than about 70%, or greater than about 80%, when compared per stage per platform.

As used herein, "superior T-cell immunosuppressive properties" refers to the capacity of a MSC to produce a greater magnitude of suppression of proliferation of T helper ($CD4^+$) lymphocytes, relative to a reference, for example as determined using an ImmunoPotency Assay.

Superior T-cell immunosuppressive properties may be about a 1% increase, about a 2% increase, about a 3% increase, about a 4% increase, about a 5% increase, about a 6% increase, about a 7% increase, about a 8% increase, about a 9% increase, about a 10% increase, about a 20% increase, about a 30% increase, about a 40% increase, about a 50% increase, about a 60% increase, about a 70% increase, about a 80% increase, about a 90% increase, about a 100%, or greater increase in T-cell immunosuppressive properties in an MSC or a population of MSCs produced according to the method disclosed herein. Alternatively, superior T-cell immunosuppressive properties may be about a 2-fold, about a 3-fold, about a 4-fold, about a 5-fold, about a 6-fold, about a 7-fold, about a 8-fold, about a 9-fold, about a 10-fold, or more increase in T-cell

immunosuppressive properties in an MSC or a population of MSCs produced according to the method disclosed herein.

A suitable ImmunoPotency Assay uses an irradiated test MSC (e.g. iPSC-MSC produced according to the method disclosed herein) and an irradiated reference sample MSC, which are plated separately at various concentrations with carboxyfluorescein succinimidyl ester-labelled leukocytes purified from healthy donor peripheral blood. T helper (CD4+) lymphocytes that represent a subset of the reference sample are stimulated by adding CD3 and CD28 antibodies. CD4 labelled T cells are enumerated using flow cytometry to assess T cell proliferation. IC50 values are reported as a function of the reference sample. A higher IC50 value indicates a greater magnitude of suppression of proliferation of T helper ($CD4^+$) lymphocytes and thus is indicative of superior T-cell immunosuppressive properties. MSC samples are irradiated prior to use in this assay to eliminate the confounding factor of their proliferative potential.

It will be appreciated by the person skilled in the art that the exact manner of administering to a subject a therapeutically effective amount of an MSC or a population of MSCs differentiated according to the present method for treating a condition, disease or disorder will be at the discretion of the medical practitioner. The mode of administration, including dosage, combination with other agents, timing and frequency of administration, and the like, may be affected by the diagnosis of a subject's likely responsiveness to treatment with the MSC or population of MSCs, as well as the subject's condition and history.

As used herein, the term "therapeutic composition" refers to a composition comprising an MSC or population of MSCs as described herein that has been formulated for administration to a subject. Preferably, the therapeutic composition is sterile. In one embodiment, the therapeutic composition is pyrogen-free.

The MSC or population of MSCs will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular type of disorder being treated, the particular subject being treated, the clinical condition of the subject, the site of administration, , the method of administration, the scheduling of

administration, possible side-effects and other factors known to medical practitioners. The therapeutically effective amount of the MSC or population of MSCs to be administered will be governed by such considerations.

The MSC or population of MSCs may be administered to a subject by any suitable method including intravenous (IV), intra-arterial, intramuscular, intraperitoneal, intracerobrospinal, subcutaneous (SC), intra-articular, intrasynovial, intrathecal, intracoronary, transendocardial, surgical implantation, topical and inhalation (e.g. intrapulmonary) routes. Most preferably, the MSC or population of MSCs is administered IV.

The term "therapeutically effective amount" refers to an amount of the MSC or population of MSCs effective to treat a condition, disease or disorder in a subject.

The terms "treat", "treating" or "treatment" refer to both therapeutic treatment and prophylactic or preventative measures, wherein the aim is to prevent or ameliorate a condition, disease or disorder in a subject or slow down (lessen) progression of a condition, disease or disorder in a subject. Subjects in need of treatment include those already with the condition, disease or disorder as well as those in which the condition, disease or disorder is to be prevented.

The terms "preventing", "prevention", "preventative" or "prophylactic" refers to keeping from occurring, or to hinder, defend from, or protect from the occurrence of a condition, a disease or disorder, including an abnormality or symptom. A subject in need of prevention may be prone to develop the condition, disease or disorder.

The term "ameliorate" or "amelioration" refers to a decrease, reduction or elimination of a condition, a disease or disorder, including an abnormality or symptom. A subject in need of treatment may already have the condition, disease or disorder, or may be prone to have the condition, disease or disorder, or may be in whom the condition, disease or disorder is to be prevented.

As used herein, the term "subject" refers to a mammal. The mammal may be a primate, particularly a human, or may be a domestic, zoo, or companion animal. Although it is particularly contemplated

that the method and its resulting MSC or population of MSCs disclosed herein are suitable for medical treatment of humans, they are also applicable to veterinary treatment, including treatment of domestic animals such as horses, cattle and sheep, companion animals such as dogs and cats, or zoo animals such as felids, canids, bovids and ungulates.

EXAMPLES

Example 1 – Reagents

Table 1. Reagents

Description	Vendor / Cat # or Ref #
DMEM/F12 Base Medium	Invitrogen / A1516901
E8 supplement	Invitrogen / A1517101
vitronectin	Life Technologies / A14700
collagen IV	Sigma / C5533
H-1152 ROCK Inhibitor	EMD Millipore / 555550
Y27632 dihydrochloride ROCK Inhibitor	Tocris / 1254
FGF2	Waisman Biomanufacturing / WC-FGF2-FP
human endothelial-SFM	Life Technologies / 11111-044
stemline II hematopoietic stem cell expansion medium	Sigma / S0192
GLUTAMAX	Invitrogen / 35050-061
insulin	Sigma / I9278
lithium chloride (LiCl)	Sigma / L4408
collagen I solution	Sigma / C2249
fibronectin	Life Technologies / 33016-015
DMEM/F12	Invitrogen / 11330032
recombinant human BMP4	Peprotech / 120-05ET
activin A	Peprotech / 120-14E
Iscove's modified Dulbecco's medium (IMDM)	Invitrogen / 12200036
Ham's F12 nutrient mix	Invitrogen / 21700075
sodium bicarbonate	Sigma / S5761
L-ascorbic acid 2-phosphate Mg^{2+}	Sigma / A8960
1-thioglycerol	Sigma / M6145
sodium selenite	Sigma / S5261
non essential amino acids	HyClone / SH30853.01
chemically defined lipid concentrate	Invitrogen / 11905031
embryo transfer grade water	Sigma / W1503
polyvinyl alcohol (PVA)	MP Bio / 151-941-83
holo-transferrin	Sigma / T0665
ES-CULT M3120	Stem Cell Technologies / 03120
STEMSPAN serum-free expansion medium (SFEM)	Stem Cell Technologies / 09650
L-ascorbic acid	Sigma / A4544
PDGF-BB	Peprotech / 110-14B

The reagents listed in Table 1 are known to the person skilled in the art and have accepted compositions, for example IMDM and Ham's F12. GLUTAMAX comprises L-alanyl-L-glutamine dipeptide, usually supplied at 200 mM in 0.85% NaCl. GLUTAMAX releases L-glutamine upon cleavage of the dipeptide bond by the cells being cultured. Chemically defined lipid concentrate comprises arachidonic acid 2 mg/L, cholesterol 220 mg/L, DL-alpha-tocopherol acetate 70 mg/L, linoleic acid 10 mg/L, linolenic acid 10 mg/L, myristic acid 10 mg/L, oleic acid 10 mg/L, palmitic acid 10 mg/L, palmitoleic acid 10 mg/L, pluronic F-68 90 g/L, stearic acid 10 mg/L, TWEEN 80® 2.2 g/L, and ethyl alcohol. H-1152 and Y27632 are highly potent, cell-permeable, selective ROCK (Rho-associated coiled coil forming protein serine/threonine kinase) inhibitors.

Table 2. IF6S medium (10X concentration)

10X IF6S	**Quantity**	**Final Concentration**
IMDM	1 package, powder for 1 L	5X
Ham's F12 nutrient mix	1 package, powder for 1 L	5X
sodium bicarbonate	4.2 g	21 mg/mL
L-ascorbic acid 2-phosphate Mg^{2+}	128 mg	640 µg/mL
1-thioglycerol	80 µL	4.6 mM
sodium selenite (0.7 mg/mL solution)	24 µL	84 ng/mL
GLUTAMAX	20 mL	10X
non essential amino acids	20 mL	10X
chemically defined lipid concentrate	4 mL	10X
embryo transfer grade water	To 200 mL	NA

Table 3. IF9S medium (1X concentration; based on IF6S)

IF9S	**Quantity**	**Final Concentration**
IF6S	5 mL	1X
polyvinyl alcohol (PVA; 20 mg/mL solution)	25 mL	10 mg/mL
holo-transferrin (10.6 mg/mL solution)	50 µL	10.6 µg/mL
insulin	100 µL	20 µg/mL
embryo transfer grade water	To 50 mL	NA

Table 4. Differentiation medium (1X concentration; based on IF9S)

Differentiation Medium	**Quantity**	**Final Concentration**
IF9S	36 mL	1X
FGF2	1.8 µg	50 ng/mL
LiCl (2M solution)	36 µL	2mM
BMP4 (100 µg/mL solution)	18 µL	50 ng/mL
Activin A (10 mg/mL solution)	5.4 µL	1.5 ng/mL

Table 5. Mesenchymal colony forming medium (1X concentration)

M-CFM	**Quantity**	**Final Concentration**
ES-CULT M3120	40 mL	40%
STEMSPAN SFEM	30 mL	30%
human endothelial-SFM	30 mL	30%
GLUTAMAX	1 mL	1X
L-ascorbic acid (250 mM solution)	100 µL	250 µM
LiCl (2M solution)	50 µL	1 mM
1-thioglycerol (100 mM solution)	100 µL	100 µM
FGF2	600 ng	20 ng/mL

Table 6. Mesenchymal serum-free expansion medium (1X concentration)

M-SFEM	**Quantity**	**Final Concentration**
human endothelial-SFM	5 L	50%
STEMLINE II HSFM	5 L	50%
GLUTAMAX	100 mL	1X
1-thioglycerol	87 µL	100 µM
FGF2	100 µg	10 ng/mL

Example 2 – Protocol for differentiating a human PSC into a MSC

1. Thawed iPSCs in E8 Complete Medium (DMEM/F12 Base Medium + E8 Supplement) + 1 µM H1152 on Vitronectin coated (0.5 µg/cm^2) plastic ware. Incubated plated iPSCs at 37°C, 5% CO_2, 20% O_2 (normoxic).
2. Expanded iPSCs three passages in E8 Complete Medium (without ROCK inhibitor) on Vitronectin coated (0.5 µg/cm^2) plastic ware and incubated at 37°C, 5% CO_2, 20% O_2 (normoxic) prior to initiating differentiation process.

3. Harvested and seeded iPSCs as single cells/small colonies at $5x10^3$ cells/cm^2 on Collagen IV coated (0.5 µg/cm^2) plastic ware in E8 Complete Medium + 10 µM Y27632 and incubated at 37°C, 5% CO_2, 20% O_2 (normoxic) for 24 h.
4. Replaced E8 Complete Medium + 10 µM Y27632 with Differentiation Medium and incubated at 37°C, 5% CO_2, 5% O_2 (hypoxic) for 48 h.
5. Harvested colony forming cells from Differentiation Medium adherent culture as a single cell suspension, transferred to M-CFM suspension culture and incubated at 37°C, 5% CO_2, 20% O_2 (normoxic) for 12 days.
6. Harvested and seeded colonies (Passage 0) on Fibronectin/Collagen I coated (0.67 µg/cm^2 Fibronectin, 1.2 µg /cm^2 Collagen I) plastic ware in M-SFEM and incubated at 37°C, 5% CO_2, 20% O_2 (normoxic) for 3 days.
7. Harvested colonies and seeded as single cells (Passage 1) at $1.3x10^4$ cells/cm^2 on Fibronectin/Collagen 1 coated plastic ware in M-SFEM and incubated at 37°C, 5% CO_2, 20% O_2 (normoxic) for 3 days.
8. Harvested and seeded as single cells (Passage 2) at $1.3x10^4$ cells/cm^2 on Fibronectin/Collagen 1 coated plastic ware in M-SFEM and incubated at 37°C, 5% CO_2, 20% O_2 (normoxic) for 3 days.
9. Harvested and seeded as single cells (Passage 3) at $1.3x10^4$ cells/cm^2 on Fibronectin/Collagen 1 coated plastic ware in M-SFEM and incubated at 37°C, 5% CO_2, 20% O_2 (normoxic) for 3 days.
10. Harvested and seeded as single cells (Passage 4) at $1.3x10^4$ cells/cm^2 on Fibronectin/Collagen 1 coated plastic ware in M-SFEM and incubated at 37°C, 5% CO_2, 20% O_2 (normoxic) for 3 days.
11. Harvested and seeded as single cells (Passage 5) at $1.3x10^4$ cells/cm^2 on Fibronectin/Collagen 1 coated plastic ware in M-SFEM and incubated at 37°C, 5% CO_2, 20% O_2 (normoxic) for 3 days.
12. Harvested as single cells and froze final product.

Two experiments (TC-A-96 and DAD-V-90) were executed to investigate the impact of supplementing M-CFM with PDGF-BB (10 ng/mL) and/or LiCl (1 mM) on T cell suppression of iPSC-MSCs. T cell suppression was evaluated generated using Waisman Biomanufacturing's ImmunoPotency Assay (IPA).

As outlined in Table 7, the following combinations of PDGF and LiCl were evaluated: PDGF+/LiCl+, PDGF-/LiCl-, PDGF+/LiCl- and PDGF-/LiCl+. Note that two different Dneg1 seed densities ($5x10^3$ cells/cm^2 and $1x10^4$ cells/cm^2) and two different concentrations of activin A (AA) in the Differentiation Medium (1X AA = 15ng/mL and 0.1X AA = 1.5ng/mL) were compared in the TC-A-96 experiment. A single Dneg1 seed density ($5x10e^3$ cells/cm^2) and activin A concentration (1.5 ng/mL) were used in the DAD-V-90 experiment. Also note that a single leukopak (LPK7) was used in the first IPA (IPA 1) and two leukopaks (LPK7 and LPK8) were used in the second IPA (IPA 2).

This assay is designed to assess the degree to which each MSC line can suppress the proliferation of T helper (CD4+) lymphocytes. Cryopreserved MSCs are tested using cryopreserved leukocytes purified from the peripheral blood of healthy individuals (peripheral blood mononucleocyte cells (PBMC) derived from Leucopaks (LPK)). As such, LPK cell population variation is expected from donor to donor. Each MSC test sample is tested against the PMBC from two different individuals for clinical grade material with the option to limit testing to a single PMBC sample for research grade material. The assay for each MSC test sample is run in conjunction with a reference standard MSC line to ensure assay integrity/ reproducibility and to normalize test samples. The assay is described in Bloom *et al. Cytotherapy*, 2015, 17(2):140-51.

In brief, test MSCs are exposed to 21 Gy of gamma irradiation. In a 48-well tissue culture plate $4x10e^5$, $2x10e^5$, $4x10e^4$, and $2x10e^4$ irradiated MSCs are plated into individual wells. PMBC are separately labelled with carboxyfluorescein succinimidyl ester. Labelled PMBC cells are plated at $4x10^5$ cells per well containing the MSCs above. This results in titrated PBMC:MSC ratios of 1:1, 1:0.5, 1:0.1, and 1:0.05. An additional well is plated with stimulated PBMCs alone, another with MSCs alone, and another 1:0.05

ratio without stimulation, all which serve as controls. Subsequently, T cell-stimulatory monoclonal antibodies, anti-human CD3-epilson and anti-human CD28 (R&D Systems, Inc., Minneapolis, MN), are added to each well.

On day four of culture, cells are harvested from individual wells. Cells from each well are incubated with allophycocyanin-labelled anti-human CD4. CD4+ cells are then analysed for proliferation via carboxyfluorescein intensity using a flow cytometer. The MSC alone control serves to gate out MSCs from co-culture wells. The PBMC alone control serves as the positive control for maximum T cell proliferation against which the degree of MSC mediated suppression is measured. The non-stimulated 1:0.05 ratio well is used to generate a negative control gate against which proliferation is measured.

From test sample ratios a best fit curve is used to generate IC50 values. The IC50 values are normalized to the reference standard (IC50 Ref Std/IC50 Test Sample). This normalized IC50 yields larger values for more potent (more suppressive) samples and smaller values for less potent samples.

Results

IC50 data presented in Table 7 show that M-CFM supplemented with LiCl, but excluding PDGF (i.e. PDGF-/LiCl+) is optimal for differentiating of iPSCs to produce iPSC-MSCs that are immunosuppressive. Furthermore, a lower concentration of activin A also improved the immunosuppression of iPSC-MSCs.

Table 7. ImmunoPotency Assay

IC50 (LPK7)	IC50 (LPK8)	Sample	PDGF	LiCl	Activin A	Seed Density (D2)
NA	not suppressive	TC-A-96-B3	+	+	0.1X (1.5ng/mL)	$5x10^3$ cells/cm^2
NA	0.17	TC-A-96-B1	+	+	1X (15ng/mL)	$5x10^3$ cells/cm^2
NA	0.17	DAD-V-90-4	+	+	0.1X (1.5ng/mL)	$5x10^3$ cells/cm^2
NA	0.19	TC-A-96-D3	+	+	0.1X (1.5ng/mL)	$1x10^4$ cells/cm^2
NA	0.36	DAD-V-90-2	+	-	0.1X (1.5ng/mL)	$5x10^3$ cells/cm^2
NA	0.57	DAD-V-90-1	-	-	0.1X (1.5ng/mL)	$5x10^3$ cells/cm^2
0.39	0.54	TC-A-96-B2	-	+	1X (15ng/mL)	$5x10^3$ cells/cm^2
0.37	0.58	TC-A-96-D2	-	+	1X (15ng/mL)	$1x10^4$ cells/cm^2
0.69	0.93	DAD-V-90-3	-	+	0.1X (1.5ng/mL)	$5x10^3$ cells/cm^2

NA – not applicable

Example 3 – MSC microRNA analysis

The MSC produced according to Example 2 underwent analysis against a microRNA (miRNA) microarray comprising 1194 miRNAs and a proprietary miRNA panel consisting of miR-127-3p, miR-145-5p, miR-181b-5p, miR-214-3p, miR-299-5p. Each of the panel of 5 miRNAs was expressed in all of 71 MSC samples, but not 94 non-MSC samples, thereby enabling classification of cells as MSC or non-MSC.

The MSC produced according to Example 2 expressed each of miR-145-5p, miR-181b-5p, and miR-214-3p, but not miR-127-3p and miR-299-5p.

A principal component analysis of the 233 miRNAs of the microarray reliably detected in the normalised data (present in at least one sample tested) generated for all the samples tested demonstrated that the MSC produced according to Example 2 was distinct from each of the other 71 MSC samples (Figure 9).

Example 4 – Alternative immunopotency assay 1

Immunopotency of MSCs is evaluated as follows: human PBMCs from various donors are pooled (to minimise inter-individual variability in immune response) in phosphate-buffered saline and

stained with carboxyfluorescein succinimidyl ester (CFSE, 2 µM) for 15 minutes at 37 °C in the dark, at a cell density of 2 x 10^7 PBMCs/mL. The reaction is stopped by adding an equal amount of RPMI-1640 medium supplemented with 10 % human blood group AB serum. 3 x 10^5 CFSE labelled PBMCs resuspended in RPMI-1640 medium supplemented with 10 % pooled human platelet lysate, 2 IU/mL preservative-free heparin (Biochrom), 2 mM L-glutamine, 10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES; Gibco), 100 IU/mL penicillin (Sigma) and 100 µg/mL streptomycin (Sigma) are then plated per well in triplicate in 96-well flat-bottomed plates (Corning). T-cell proliferation is determined using a Gallios 10-color flow cytometer and the Kaluza G1.0 software (both Coulter). Viable 7-aminoactinomycin-D-excluding (7-AAD-; BD Pharmingen) CD3-APC+ (eBioscience) T cells are analysed after 4 to 7 days. Proliferation kinetics and population distribution are analysed using Modfit 4.1 software (Verity).

Example 5 – Alternative immunopotency assay 2

Immunopotency of MSCs is evaluated as follows: T helper (CD4+) lymphocytes are stained with CellTrace violet (CTV; Invitrogen) according to the manufacturer's instructions and then stimulated with anti-CD3/CD28-coated beads (Dynabeads, Invitrogen) at a T-cell/bead ratio of 5:1 in 96-well U-bottomed plates. Responder CD4 T cells are then incubated with irradiated (at 100 Gy) Karpas 299 cells (K299 cells; Sigma) as a reference standard, or MSCs. The co-cultured cells are incubated at 37°C in 5% CO_2 in RPMI-1640 medium for 72 h. The cells are then washed with AnnexinV binding buffer (BD Biosciences) and stained with Annexin Vefluorescein isothiocyanate or APC (BD Biosciences) for 15 min in the dark at room temperature. After this incubation, the cells are stained with propidium iodide (PI) (Molecular Probes) and then immediately acquired on a LSRII Fortessa (BD Biosciences). Collected data are analysed with the use of FlowJo software (version 8.8.6; Tree Star). The viability is measured by the population of Annexin Venegative and PI-negative T cells. This proportion of viable cells is analysed for CTV dim (% proliferation). Suppression of T-cell proliferation is calculated by means of the equation: % Suppression

= 100 - (a/b * 100), where a is the percentage proliferation in the presence of suppressor cells and b is the percentage proliferation in the absence of suppressor cells.

Example 6 - Treatment of myocardial infarction

Mesenchymal stem cells (MSCs) of the present disclosure were used in an experimental rat model of myocardial infarction (heart attack) to repair the rat heart after a heart attack.

Cardiac function and scar size were assessed over a 28 day period after a heart attack was induced in a total of 11 rats. Four animals were treated with the MSCs of the disclosure, three animals were treated with bone marrow-derived MSCs, and a further four animals received a placebo/ vehicle control (figure 10).

Assessment of cell engraftment (figures 11 and 12) showed:

- No evidence of cell engraftment using human nuclei and human mitochondria staining at day 28 in the BM-MSC and PSC-MSC groups

Functional assessment (figures 13 and 14) using fractional shortening (FS %) at day 28 compared to day 0 showed:

- minimal/ no change in LV contractility in vehicle group
- LV contractility in BM MSC group
- LV contractility in PSC-MSC group

Assessment of scar size (figure 15) showed:

- Apparent reduction in scar size on day 28 in PSC-MSC group (picosirius red) compared to vehicle and BM-MSCs group.

Assessment of angiogenesis (figure 16) showed:

- No obvious difference in size and number of vessels between groups (vWF staining).

The results showed that cardiac function was improved and scar size was reduced in the MSC recipients at day 28 compared to animals in both of the other groups. The results showed that the MSCs of the disclosure caused a substantial functional and structural improvement after a heart attack.

In a modification of the study of example 6 as shown in figure 17, arrhythmia will be assessed using programmed ventricular stimulation (EPS).

Example 7 – Treatment of GvHD

Animals were randomly assigned to treatment groups, and in all cases, the relevant treatment was injected via the tail vein. On day 0, six-week old female NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ (NSG) mice were lightly irradiated with 2.5Gy, then rested for 4 hours. GvHD was induced by intravenous (tail vein) transfer of 10 million human PBMCs. Following disease induction, mice were housed under pathogen-free conditions in micro-isolator cages and received acidified, antibiotic-supplemented water throughout the duration of the experimental procedures. The study design is summarised in Table 8.

Table 8. Summary of GvHD Study Design

Group	Group description	PBMC Dose[1]	MSC Dose	n
Parts 1 and 2 – Survival studies				
1A	Irradiation controls (no GvHD)	0	0	8
1B	GvHD controls[2]	$10x10^6$	0	8
1C	Single dose controls (no GvHD)	0	$2x10^6$ on d14	8
1D	MSC treated – single dose	$10x\ 10^6$	$2x10^6$ on d14	12
2A	Dual dose controls (no GvHD)	0	$2x10^6$ on d14 & d18	8
2B	MSC treated – dual dose	$10x10^6$	$2x10^6$ on d14 & d18	12

1. All animals were lightly irradiated (2.5Gy) on Day 0. PBMCs were administered 4 hours after irradiation to induce GvHD (if applicable).

2. GvHD controls received phosphate buffered saline (PBS) instead of MSC treatment.

Animals were assessed on a daily basis for GvHD signs/ symptoms – i.e. weight loss, posture, activity, fur texture and skin integrity – as described in Table 9. An overall GvHD score was calculated by assigning 1 point for each Grade 1 sign/ symptom and 2 points for each Grade 2 sign/ symptom. Once a GvHD score of 8 or higher was reached in any animal, it was euthanised.

Table 9. GvHD grading

Symptoms	Grade 0	Grade 1	Grade 2
Weight loss	<10%	>10%; <20%	>20%
Posture	Normal	Hunching noted only at rest	Severe hunching and/or impairment of movement
Activity	Normal	Mild-moderate decrease	Stationary unless stimulated
Fur texture	Normal	Mild to moderate ruffling	Severe ruffling and poor grooming
Skin integrity	Normal	Scaling of paws/tail	Obvious areas of denuded skin

The primary endpoint was duration of survival.

Results

iPSC-MSCs were thawed, washed, and resuspended in sterile PBS. Two million iPSC-MSCs were administered to relevant animals through the tail vein on day +14 (d14, single dose regimen) or on days +14 and +18 (d14, d18, dual-dose regimen).

The severity of GvHD was assessed using a standardised scoring system, which included five different criteria: weight loss, posture, activity, fur texture, and skin integrity. Mice were evaluated daily and scored from 0 (the least severe) to 2 (the most severe) for each criterion. Clinical scores were generated by adding scores for the five criteria. When a clinical score of "8" was reached, mice were removed from the study and humanely euthanised. The day of removal from the study was recorded as the day of lethal GvHD induction. Survival benefit was determined using Kaplan-Meier analysis with an applied log-rank test. p values of ≤ 0.05 were considered significantly different.

Animals receiving single- or dual-dose regimens of iPSC-MSCs showed significant relief from weight loss typically associated with this pre-clinical model (Figure 18).

Mice treated with single- or dual-dose regimens showed significant attenuation of disease symptoms, with further significant differences in symptoms noted between single- and dual-dose treatments on days +24 and +25 post-GvHD-induction (Figure 19).

In survival studies, single- and dual-dose treatments conferred significant survival benefits over GVHD controls ($p<0.0001$). Survival of animals receiving dual-dose regimens was slightly improved over single-dose-treated animals, although this increase did not reach statistical significance ($p=0.0715$). (Figure 20)

From these survival studies, we conclude that CYMERUS™ iPSC-MSCs protect mice from weight loss, significantly attenuate disease severity, and provide a robust survival benefit when administered either in single- or dual-dose treatments, in a pre-clinical model of GvHD.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method for producing a mesenchymal stem cell (MSC), the method comprising:

(a) culturing a primitive mesodermal cell in a mesenchymal-colony forming medium (M-CFM) comprising LiCl and FGF2, but excluding PDGF, BMP4 and Activin A, under normoxic conditions for sufficient time for a mesenchymal colony to form; and

(b) culturing the mesenchymal colony of (a) adherently to produce the MSC,

wherein the MSC of (b) has superior T-cell immunosuppressive properties relative to an MSC produced by a method comprising:

(a') culturing a primitive mesodermal cell in a medium comprising FGF2 and PDGF, but excluding LiCl, under normoxic conditions for sufficient time for a mesenchymal colony to form; and

(b') culturing the mesenchymal colony of (a') adherently to produce the MSC.

2. The method of claim 1, wherein the primitive mesodermal cell is a primitive mesodermal cell with mesenchymoangioblast (MCA) potential.

3. The method of claim 2, wherein the primitive mesodermal cell with MCA potential has a ^{EMH}lin^{-}KDR^{+}APLNR^{+}PDGFRalpha^{+} phenotype.

4. The method of claim 1, wherein sufficient time for the mesenchymal colony to form is about 8 days to about 14 days,

Date Reçue/Date Received 2022-07-19

about 10 days to about 14 days, about 11 to about 13 days, or about 12 days.

5. The method of claim 1, wherein the T-cell immunosuppressive properties of the MSC are determined relative to an MSC produced from a primitive mesodermal cell differentiated from a pluripotent stem cell (PSC) in a differentiation medium comprising FGF2, BMP4, Activin A, and LiCl under hypoxic conditions for about two days.

6. The method of claim 1, wherein T-cell immunosuppressive properties comprise suppression of proliferation of T helper ($CD4^+$) lymphocytes.

7. The method of claim 1, wherein the M-CFM comprises about 1 mM LiCl.

8. The method of claim 1, wherein the M-CFM comprises about 5 ng/mL to about 100 ng/mL FGF2, about 10 ng/mL to about 50 ng/mL FGF2, about 10 ng/mL FGF2, or about 20 ng/mL FGF2.

9. The method of claim 1, wherein the M-CFM comprises about 1 mM LiCl and about 10 ng/mL FGF2, or about 1 mM LiCl and about 20 ng/mL FGF2.

10. The method of claim 1, further comprising, prior to culturing the primitive mesodermal cell, differentiating a pluripotent stem cell (PSC) into a primitive mesodermal cell comprising:

culturing the PSC in a differentiation medium comprising FGF2, BMP4, Activin A, and LiCl under hypoxic conditions for about two days to form the primitive mesoderm; and

Date Reçue/Date Received 2022-07-19

replacing the differentiation medium with the M-CFM comprising LiCl and FGF2, but excluding PDGF, BMP4 and Activin A.

11. The method of claim 1, wherein the cells are human.

12. The method of claim 10, wherein the PSC is an induced PSC (iPSC).

13. The method of claim 10, wherein the differentiation medium comprises:

about 10 ng/mL to about 50 ng/mL FGF2, or about 50 ng/mL FGF2;

about 50 ng/mL to about 250 ng/mL BMP4, or about 50 ng/mL BMP4;

about 1 ng/mL to about 15 ng/mL Activin A, about 10 ng/mL to about 15 ng/mL Activin A, about 12.5 ng/mL Activin A, or about 1.5 ng/mL Activin A; and/or

about 1 mM to about 2 mM LiCl.

14. The method of claim 10, wherein the differentiation medium comprises:

about 50 ng/mL FGF2;

about 50 ng/mL BMP4;

about 1.5 ng/mL Activin A; and

about 2 mM LiCl.

15. The method of claim 1, comprising culturing on collagen IV and/or tenascin C.

16. The method of claim 1, further comprising passaging the mesenchymal colony and/or MSC.

Date Reçue/Date Received 2022-07-19

17. The method of claim 16, wherein passaging comprises:

culturing the mesenchymal colony and/or MSC for about 3 days; or

culturing the mesenchymal colony and/or MSC on fibronectin and/or collagen I; for

1, 2, 3, 4, 5, or 6 passages.

Date Reçue/Date Received 2022-07-19

FIGURES

GLECDGKVNI CCKKQFFVSF KDIGWNDWII APSGYHANYC EGECPSHIAG TSGSSLSFHS
TVINHYRMRG HSPFANLKSC CVPTKLRPMS MLYYDDGQNI IKKDIQNMIV EECGCS

Figure 1.

KKNKNCRRHS LYVDFSDVGW NDWIVAPPGY QAFYCHGDCP FPLADHLNST NHAIVQTLVN
SVNSSIPKAC CVPTELSAIS MLYLDEYDKV VLKNYQEMVV EGCGCR

Figure 2.

QLSYGYDEKS TGGISVPGPM GPSGPRGLPG PPGAPGPQGF QGPPGEPGEP GASGPMGPRG
PPGPPGKNGD DGEAGKPGRP GERGPPGPQG ARGLPGTAGL PGMKGHRGFS GLDGAKGDAG
PAGPKGEPGS PGENGAPGQM GPRGLPGERG RPGAPGPAGA RGNDGATGAA GPPGPTGPAG
PPGFPGAVGA KGEAGPQGPR GSEGPQGVRG EPGPPGPAGA AGPAGNPGAD GQPGAKGANG
APGIAGAPGF PGARGPSGPQ GPGGPPGPKG NSGEPGAPGS KGDTGAKGEP GPVGVQGPPG
PAGEEGKRGA RGEPGPTGLP GPPGERGGPG SRGFPGADGV AGPKGPAGER GSPGPAGPKG
SPGEAGRPGE AGLPGAKGLT GSPGSPGPDG KTGPPGPAGQ DGRPGPPGPP GARGQAGVMG
FPGPKGAAGE PGKAGERGVP GPPGAVGPAG KDGEAGAQGP PGPAGPAGER GEQGPAGSPG
FQGLPGPAGP PGEAGKPGEQ GVPGDLGAPG PSGARGERGF PGERGVQGPP GPAGPRGANG
APGNDGAKGD AGAPGAPGSQ GAPGLQGMPG ERGAAGLPGP KGDRGDAGPK GADGSPGKDG
VRGLTGPIGP PGPAGAPGDK GESGPSGPAG PTGARGAPGD RGEPGPPGPA GFAGPPGADG
QPGAKGEPGD AGAKGDAGPP GPAGPAGPPG PIGNVGAPGA KGARGSAGPP GATGFPGAAG
RVGPPGPSGN AGPPGPPGPA GKEGGKGPRG ETGPAGRPGE VGPPGPPGPA GEKGSPGADG
PAGAPGTPGP QGIAGQRGVV GLPGQRGERG FPGLPGPSGE PGKQGPSGAS GERGPPGPMG
PPGLAGPPGE SGREGAPGAE GSPGRDGSPG AKGDRGETGP AGPPGAPGAP GAPGPVGPAG
KSGDRGETGP AGPAGPVGPV GARGPAGPQG PRGDKGETGE QGDRGIKGHR GFSGLQGPPG
PPGSPGEQGP SGASGPAGPR GPPGSAGAPG KDGLNGLPGP IGPPGPRGRT GDAGPVGPPG
PPGPPGPPGP PSAGFDFSFL PQPPQEKAHD GGRYYRA

Figure 3.

GERGFPGIPG TPGPPGLPGL QGPVGPPGFT GPPGPPGPPG PPGEKGQMGL SFQGPKGDKG
DQGVSGPPGV PGQAQVQEKG DFATKGEKGQ KGEPGFQGMP GVGEKGEPGK PGPRGKPGKD
GDKGEKGSPG FPGEPGYPGL IGRQGPQGEK GEAGPPGPPG IVIGTGPLGE KGERGYPGTP
GPRGEPGPKG FPGLPGQPGP PGLPVPGQAG APGFPGERGE KGDRGFPGTS LPGPSGRDGL
PGPPGSPGPP GQPGYTNGIV ECQPGPPGDQ GPPGIPGQPG FIGEIGEKGQ KGESCLICDI
DGYRGPPGPQ GPPGEIGFPG QPGAKGDRGL PGRDGVAGVP GPQGTPGLIG QPGAKGEPGE
FYFDLRLKGD KGDPGFPGQP GMPGRAGSPG RDGHPGLPGP KGSPGSVGLK GERGPPGGVG
FPGSRGDTGP PGPPGYGPAG PIGDKGQAGF PGGPGSPGLP GPKGEPGKIV PLPGPPGAEG
LPGSPGFPGP QGDRGFPGTP GRPGLPGEKG AVGQPGIGFP GPPGPKGVDG LPGDMGPPGT
PGRPGFNGLP GNPGVQGQKG EPGVGLPGLK GLPGLPGIPG TPGEKGSIGV PGVPGEHGAI
GPPGLQGIRG EPGPPGLPGS VGSPGVPGIG PPGARGPPGG QGPPGLSGPP GIKGEKGFPG
FPGLDMPGPK GDKGAQGLPG ITGQSGLPGL PGQQGAPGIP GFPGSKGEMG VMGTPGQPGS
PGPVGAPGLP GEKGDHGFPG SSGPRGDPGL KGDKGDVGLP GKPGSMDKVD MGSMKGQKGD
QGEKGQIGPI GEKGSRGDPG TPGVPGKDGQ AGQPGQPGPK GDPGISGTPG APGLPGPKGS
VGGMGLPGTP GEKGVPGIPG PQGSPGLPGD KGAKGEKGQA GPPGIGIPGL RGEKGDQGIA
GFPGSPGEKG EKGSIGIPGM PGSPGLKGSP GSVGYPGSPG LPGEKGDKGL PGLDGIPGVK
GEAGLPGTPG PTGPAGQKGE PGSDGIPGSA GEKGEPGLPG RGFPGFPGAK GDKGSKGEVG
FPGLAGSPGI PGSKGEQGFM GPPGPQGQPG LPGSPGHATE GPKGDRGPQG QPGLPGLPGP
MGPPGLPGID GVKGDKGNPG WPGAPGVPGP KGDPGFQGMP GIGGSPGITG SKGDMGPPGV
PGFQGPKGLP GLQGIKGDQG DQGVPGAKGL PGPPGPPGPY DIIKGEPGLP GPEGPPGLKG
LQGLPGPKGQ QGVTGLVGIP GPPGIPGFDG APGQKGEMGP AGPTGPRGFP GPPGPDGLPG
SMGPPGTPSV DHGFLVTRHS QTIDDPQCPS GTKILYHGYS LLYVQGNERA HGQDLGTAGS
CLRKFSTMPF LFCNINNVCN FASRNDYSYW LSTPEPMPMS MAPITGENIR PFISRCAVCE
APAMVMAVHS QTIQIPPCPS GWSSLWIGYS FVMHTSAGAE GSGQALASPG SCLEEFRSAP
FIECHGRGTC NYYANAYSFW LATIERSEMF KKPTPSTLKA GELRTHVSRC QVCMRRT

Figure 4.

MVGVGGGDVE DVTPRPGGCQ ISGRGARGCN GIPGAAAWEA ALPRRRPRRH PSVNPRSRAA
GSPRTRGRRT EERPSGSRLG DRGRGRALPG GRLGGRGRGR APERVGGRGR GRGTAAPRAA
PAARGSRPGP AGTMAAGSIT TLPALPEDGG SGAFPPGHFK DPKRLYCKNG GFFLRIHPDG
RVDGVREKSD PHIKLQLQAE ERGVVSIKGV CANRYLAMKE DGRLLASKCV TDECFFFERL
ESNNYNTYRS RKYTSWYVAL KRTGQYKLGS KTGPGQKAIL FLPMSAKS

Figure 5.

QAQQMVQPQS PVAVSQSKPG CYDNGKHYQI NQQWERTYLG NALVCTCYGG SRGFNCESKP
EAEETCFDKY TGNTYRVGDT YERPKDSMIW DCTCIGAGRG RISCTIANRC HEGGQSYKIG
DTWRRPHETG GYMLECVCLG NGKGEWTCKP IAEKCFDHAA GTSYVVGETW EKPYQGWMMV
DCTCLGEGSG RITCTSRNRC NDQDTRTSYR IGDTWSKKDN RGNLLQCICT GNGRGEWKCE
RHTSVQTTSS GSGPFTDVRA AVYQPQPHPQ PPPYGHCVTD SGVVYSVGMQ WLKTQGNKQM
LCTCLGNGVS CQETAVTQTY GGNSNGEPCV LPFTYNGRTF YSCTTEGRQD GHLWCSTTSN
YEQDQKYSFC TDHTVLVQTR GGNSNGALCH FPFLYNNHNY TDCTSEGRRD NMKWCGTTQN
YDADQKFGFC PMAAHEEICT TNEGVMYRIG DQWDKQHDMG HMMRCTCVGN GRGEWTCIAY
SQLRDQCIVD DITYNVNDTF HKRHEEGHML NCTCFGQGRG RWKCDPVDQC QDSETGTFYQ
IGDSWEKYVH GVRYQCYCYG RGIGEWHCQP LQTYPSSSGP VEVFITETPS QPNSHPIQWN
APQPSHISKY ILRWRPKNSV GRWKEATIPG HLNSYTIKGL KPGVVYEGQL ISIQQYGHQE
VTRFDFTTTS TSTPVTSNTV TGETTPFSPL VATSESVTEI TASSFVVSWV SASDTVSGFR
VEYELSEEGD EPQYLDLPST ATSVNIPDLL PGRKYIVNVY QISEDGEQSL ILSTSQTTAP
DAPPDPTVDQ VDDTSIVVRW SRPQAPITGY RIVYSPSVEG SSTELNLPET ANSVTLSDLQ
PGVQYNITIY AVEENQESTP VVIQQETTGT PRSDTVPSPR DLQFVEVTDV KVTIMWTPPE
SAVTGYRVDV IPVNLPGEHG QRLPISRNTF AEVTGLSPGV TYYFKVFAVS HGRESKPLTA
QQTTKLDAPT NLQFVNETDS TVLVRWTPPR AQITGYRLTV GLTRRGQPRQ YNVGPSVSKY
PLRNLQPASE YTVSLVAIKG NQESPKATGV FTTLQPGSSI PPYNTEVTET TIVITWTPAP
RIGFKLGVRP SQGGEAPREV TSDSGSIVVS GLTPGVEYVY TIQVLRDGQE RDAPIVNKVV
TPLSPPTNLH LEANPDTGVL TVSWERSTTP DITGYRITTT PTNGQQGNSL EEVVHADQSS
CTFDNLSPGL EYNVSVYTVK DDKESVPISD TIIPEVPQLT DLSFVDITDS SIGLRWTPLN
SSTIIGYRIT VVAAGEGIPI FEDFVDSSVG YYTVTGLEPG IDYDISVITL INGGESAPTT
LTQQTAVPPP TDLRFTNIGP DTMRVTWAPP PSIDLTNFLV RYSPVKNEED VAELSISPSD
NAVVLTNLLP GTEYVVSVSS VYEQHESTPL RGRQKTGLDS PTGIDFSDIT ANSFTVHWIA
PRATITGYRI RHHPEHFSGR PREDRVPHSR NSITLTNLTP GTEYVVSIVA LNGREESPLL
IGQQSTVSDV PRDLEVVAAT PTSLLISWDA PAVTVRYYRI TYGETGGNSP VQEFTVPGSK
STATISGLKP GVDYTITVYA VTGRGDSPAS SKPISINYRT EIDKPSQMQV TDVQDNSISV
KWLPSSSPVT GYRVTTTPKN GPGPTKTKTA GPDQTEMTIE GLQPTVEYVV SVYAQNPSGE
SQPLVQTAVT NIDRPKGLAF TDVDVDSIKI AWESPQGQVS RYRVTYSSPE DGIHELFPAP
DGEEDTAELQ GLRPGSEYTV SVVALHDDME SQPLIGTQST AIPAPTDLKF TQVTPTSLSA
QWTPPNVQLT GYRVRVTPKE KTGPMKEINL APDSSSVVVS GLMVATKYEV SVYALKDTLT
SRPAQGVVTT LENVSPPRRA RVTDATETTI TISWRTKTET ITGFQVDAVP ANGQTPIQRT
IKPDVRSYTI TGLQPGTDYK IYLYTLNDNA RSSPVVIDAS TAIDAPSNLR FLATTPNSLL
VSWQPPRARI TGYIIKYEKP GSPPREVVPR PRPGVTEATI TGLEPGTEYT IYVIALKNNQ
KSEPLIGRKK TDELPQLVTL PHPNLHGPEI LDVPSTVQKT PFVTHPGYDT GNGIQLPGTS
GQQPSVGQQM IFEEHGFRRT TPPTTATPIR HRPRPYPPNV GQEALSQTTI SWAPFQDTSE
YIISCHPVGT DEEPLQFRVP GTSTSATLTG LTRGATYNII VEALKDQQRH KVREEVVTVG
NSVNEGLNQP TDDSCFDPYT VSHYAVGDEW ERMSESGFKL LCQCLGFGSG HFRCDSSRWC
HDNGVNYKIG EKWDRQGENG QMMSCTCLGN GKGEFKCDPH EATCYDDGKT YHVGEQWQKE
YLGAICSCTC FGGQRGWRCD NCRRPGGEPS PEGTTGQSYN QYSQRYHQRT NTNVNCPIEC
FMPLDVQADR EDSRE

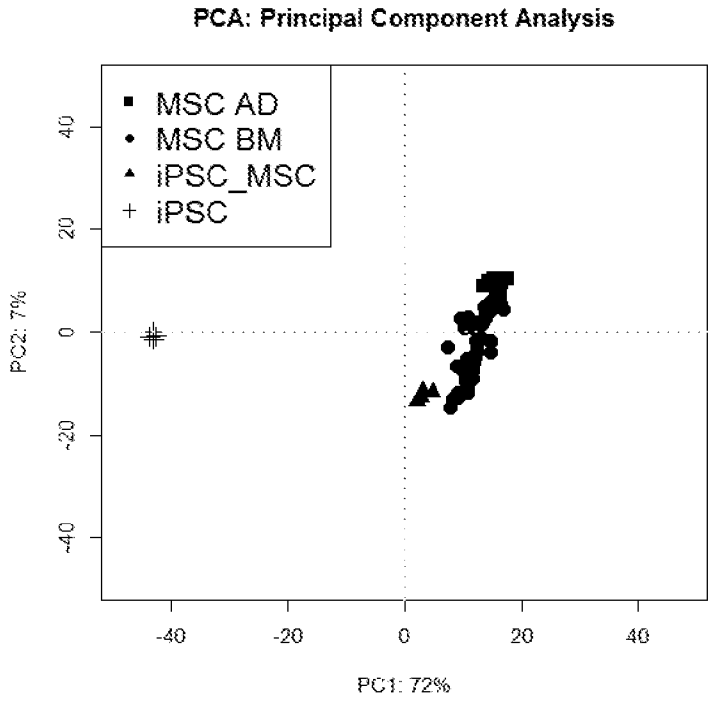
Figure 6.

SLGSLTIAEP AMIAECKTRT EVFEISRRLI DRTNANFLVW PPCVEVQRCS GCCNNRNVQC
RPTQVQLRPV QVRKIEIVRK KPIFKKATVT LEDHLACKCE TVAAARPVT

Figure 7.

GVLKKVIRHK RQSGVNATLP EENQPVVFNH VYNIKLPVGS QCSVDLESAS GEKDLAPPSE
PSESFQEHTV DGENQIVFTH RINIPRRACG CAAAPDVKEL LSRLEELENL VSSLREQCTA
GAGCCLQPAT GRLDTRPFCS GRGNFSTEGC GCVCEPGWKG PNCSEPECPG NCHLRGRCID
GQCICDDGFT GEDCSQLACP SDCNDQGKCV NGVCICFEGY AGADCSREIC PVPCSEEHGT
CVDGLCVCHD GFAGDDCNKP LCLNNCYNRG RCVENECVCD EGFTGEDCSE LICPNDCFDR
GRCINGTCYC EEGFTGEDCG KPTCPHACHT QGRCEEGQCV CDEGFAGVDC SEKRCPADCH
NRGRCVDGRC ECDDGFTGAD CGELKCPNGC SGHGRCVNGQ CVCDEGYTGE DCSQLRCPND
CHSRGRCVEG KCVCEQGFKG YDCSDMSCPN DCHQHGRCVN GMCVCDDGYT GEDCRDRQCP
RDCSNRGLCV DGQCVCEDGF TGPDCAELSC PNDCHGQGRC VNGQCVCHEG FMGKDCKEQR
CPSDCHGQGR CVDGQCICHE GFTGLDCGQH SCPSDCNNLG QCVSGRCICN EGYSGEDCSE
VSPPKDLVVT EVTEETVNLA WDNEMRVTEY LVVYTPTHEG GLEMQFRVPG DQTSTIIQEL
EPGVEYFIRV FAILENKKSI PVSARVATYL PAPEGLKFKS IKETSVEVEW DPLDIAFETW
EIIFRNMNKE DEGEITKSLR RPETSYRQTG LAPGQEYEIS LHIVKNNTRG PGLKRVTTTR
LDAPSQIEVK DVTDTTALIT WFKPLAEIDG IELTYGIKDV PGDRTTIDLT EDENQYSIGN
LKPDTEYEVS LISRRGDMSS NPAKETFTTG LDAPRNLRRV SQTDNSITLE WRNGKAAIDS
YRIKYAPISG GDHAEVDVPK SQQATTKTTL TGLRPGTEYG IGVSAVKEDK ESNPATINAA
TELDTPKDLQ VSETAETSLT LLWKTPLAKF DRYRLNYSLP TGQWVGVQLP RNTTSYVLRG
LEPGQEYNVL LTAEKGRHKS KPARVKASTE QAPELENLTV TEVGWDGLRL NWTAADQAYE
HFIIQVQEAN KVEAARNLTV PGSLRAVDIP GLKAATPYTV SIYGVIQGYR TPVLSAEAST
GETPNLGEVV VAEVGWDALK LNWTAPEGAY EYFFIQVQEA DTVEAAQNLT VPGGLRSTDL
PGLKAATHYT ITIRGVTQDF STTPLSVEVL TEEVPDMGNL TVTEVSWDAL RLNWTTPDGT
YDQFTIQVQE ADQVEEAHNL TVPGSLRSME IPGLRAGTPY TVTLHGEVRG HSTRPLAVEV
VTEDLPQLGD LAVSEVGWDG LRLNWTAADN AYEHFVIQVQ EVNKVEAAQN LTLPGSLRAV
DIPGLEAATP YRVSIYGVIR GYRTPVLSAE ASTAKEPEIG NLNVSDITPE SFNLSWMATD
GIFETFTIEI IDSNRLLETV EYNISGAERT AHISGLPPST DFIVYLSGLA PSIRTKTISA
TATTEALPLL ENLTISDINP YGFTVSWMAS ENAFDSFLVT VVDSGKLLDP QEFTLSGTQR
KLELRGLITG IGYEVMVSGF TQGHQTKPLR AEIVTEAEPE VDNLLVSDAT PDGFRLSWTA
DEGVFDNFVL KIRDTKKQSE PLEITLLAPE RTRDITGLRE ATEYEIELYG ISKGRRSQTV
SAIATTAMGS PKEVIFSDIT ENSATVSWRA PTAQVESFRI TYVPITGGTP SMVTVDGTKT
QTRLVKLIPG VEYLVSIIAM KGFEESEPVS GSFTTALDGP SGLVTANITD SEALARWQPA
IATVDSYVIS YTGEKVPEIT RTVSGNTVEY ALTDLEPATE YTLRIFAEKG PQKSSTITAK
FTTDLDSPRD LTATEVQSET ALLTWRPPRA SVTGYLLVYE SVDGTVKEVI VGPDTTSYSL
ADLSPSTHYT AKIQALNGPL RSNMIQTIFT TIGLLYPFPK DCSQAMLNGD TTSGLYTIYL
NGDKAEALEV FCDMTSDGGG WIVFLRRKNG RENFYQNWKA YAAGFGDRRE EFWLGLDNLN
KITAQGQYEL RVDLRDHGET AFAVYDKFSV GDAKTRYKLK VEGYSGTAGD SMAYHNGRSF
STFDKDTDSA ITNCALSYKG AFWYRNCHRV NLMGRYGDNN HSQGVNWFHW KGHEHSIQFA
EMKLRPSNFR NLEGRRKRA

Figure 8.



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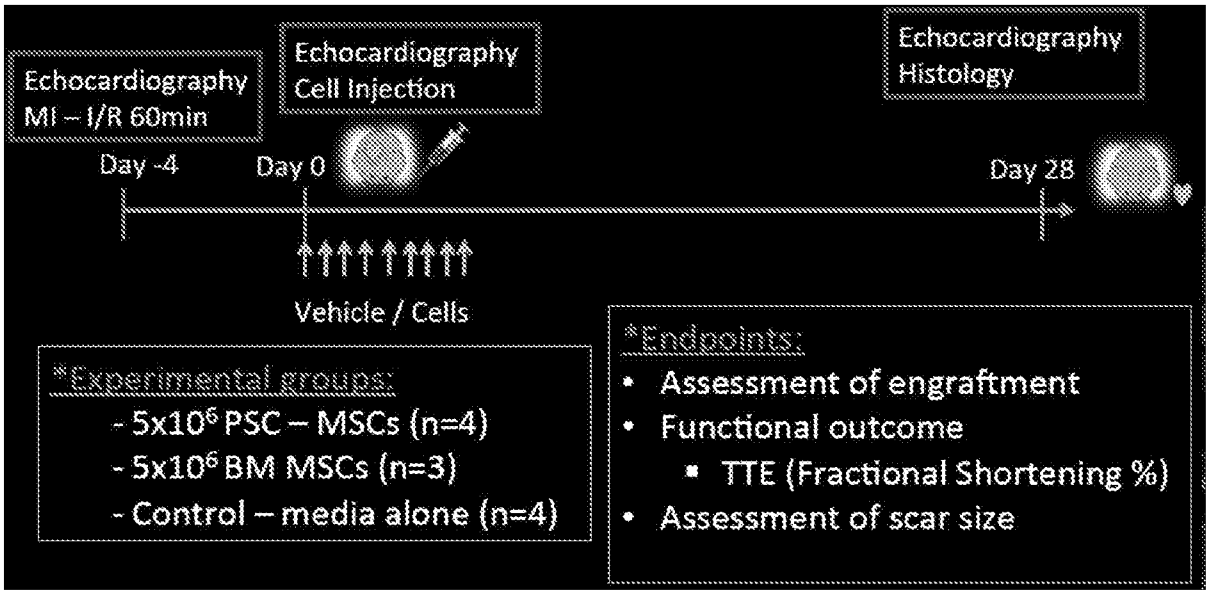


Figure 10.

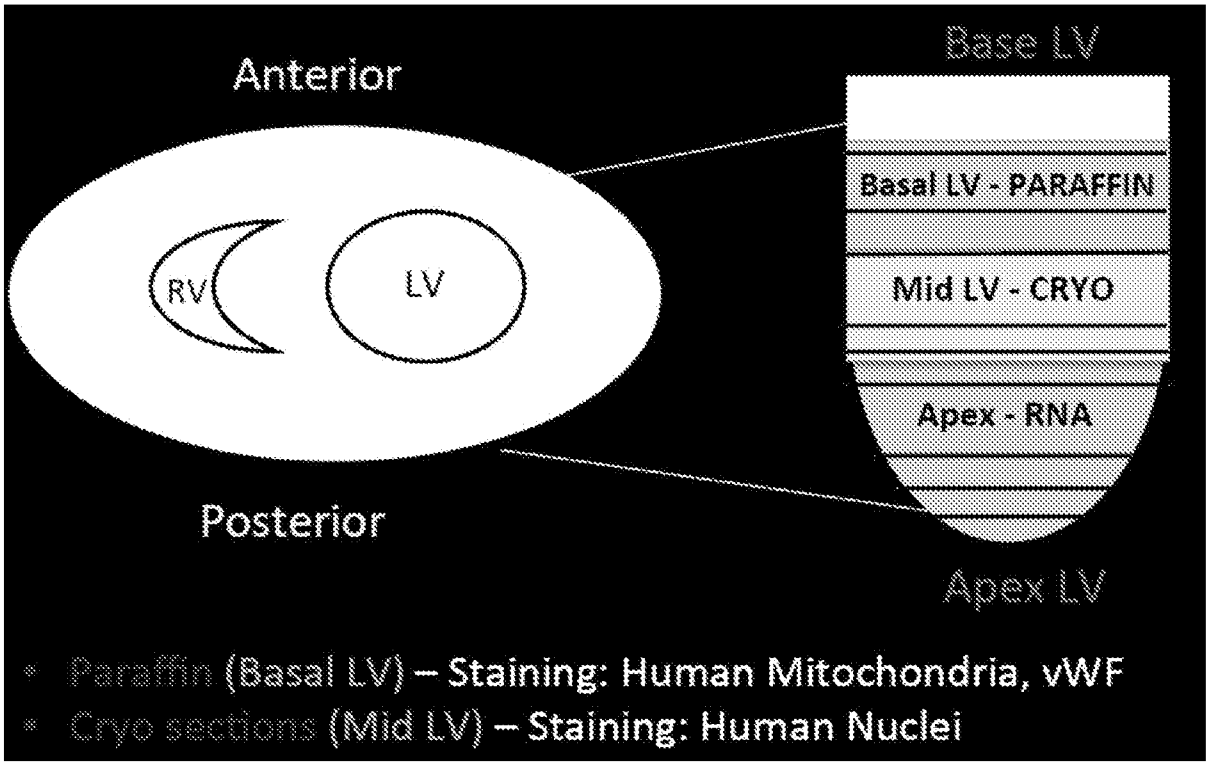
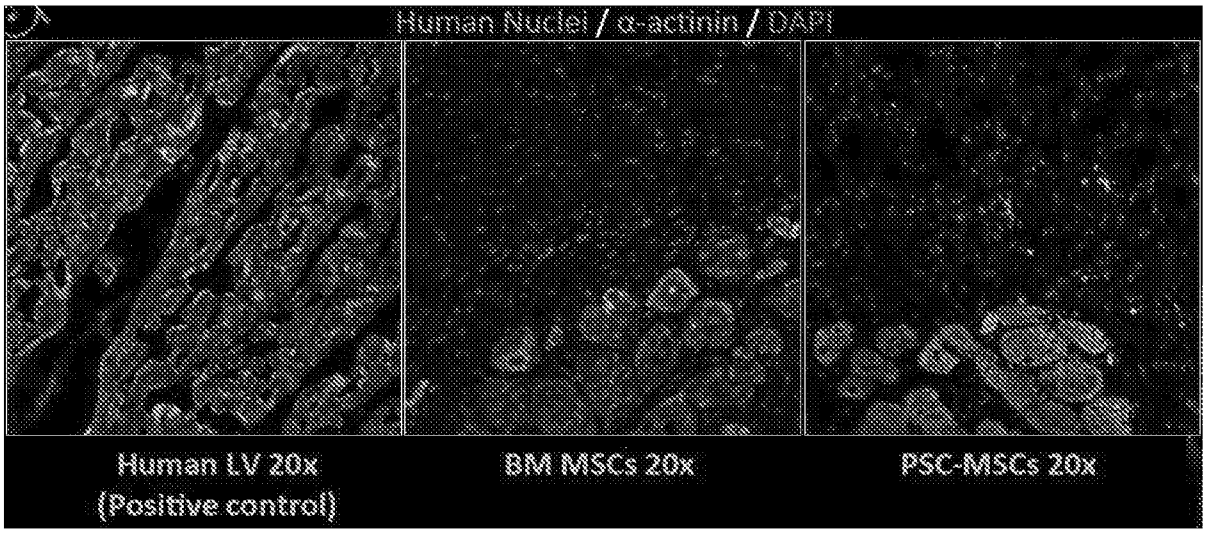


Figure 11.



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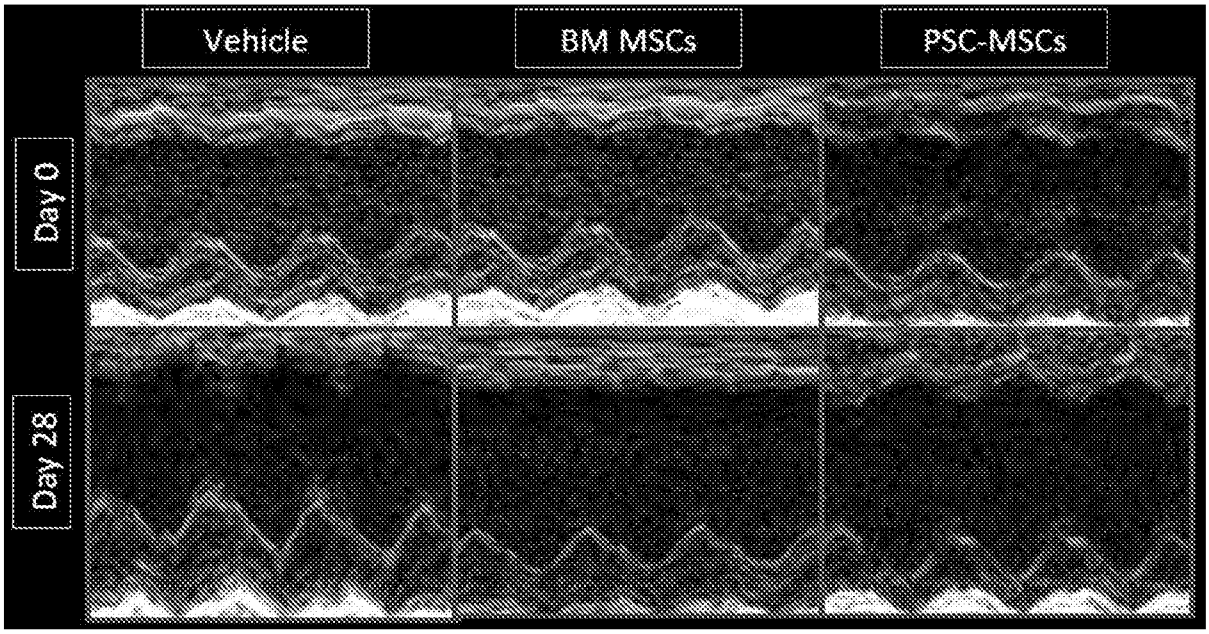
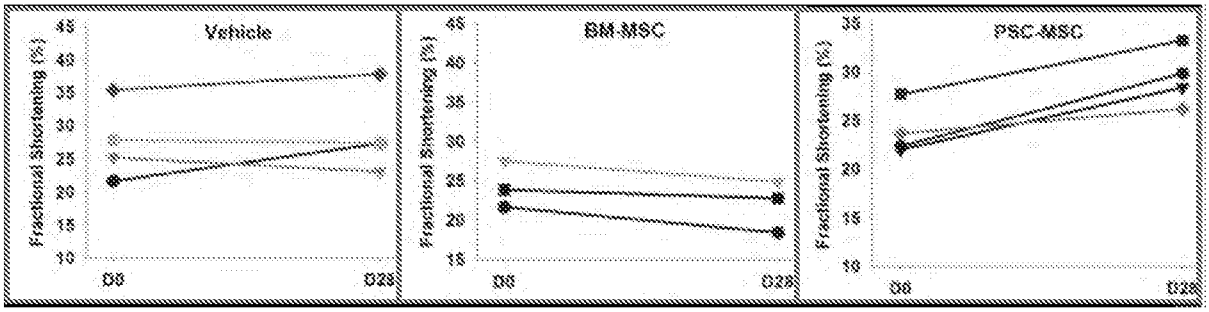


Figure 13.



Randomisation	Day -4 FS (%) (mean ±SE)	Day 0 FS (%) (mean ±SE)	Day 28 FS (%) (mean ±SE)	δEF (D0 to D28) (%)
Vehicle (n=4)	49.85±1.13	27.56±2.92	28.95±3.14	1.39±1.72
BM MSC (n=3)	49.57±0.05	24.35±1.70	22.03±1.90	-2.33±0.65
PSC-MSC (n=4)	50.67±0.61	23.95±1.31	29.43±1.47	5.48±1.06*

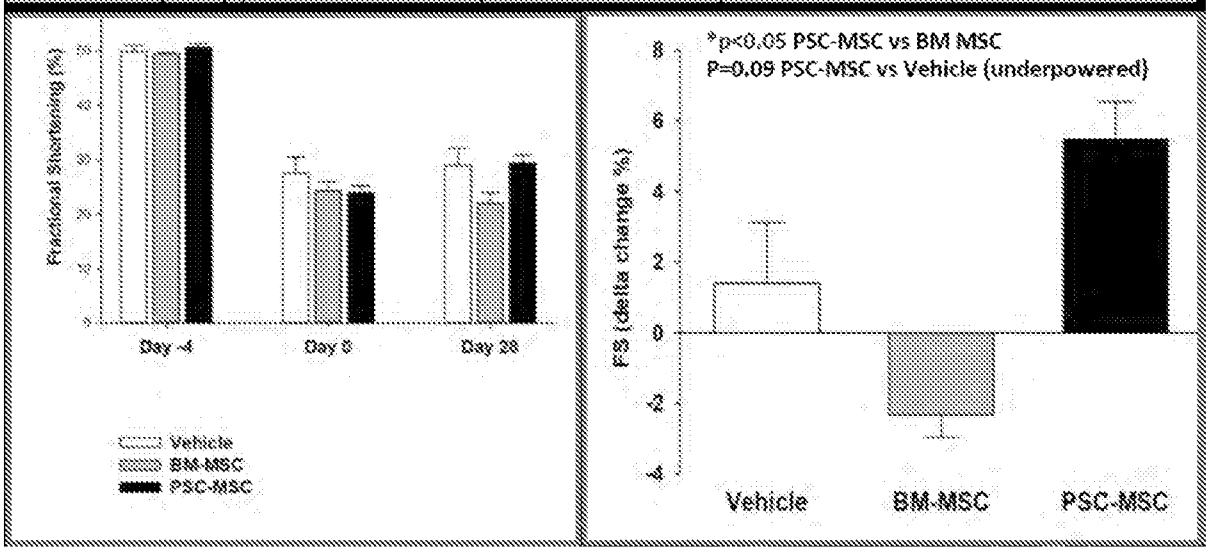


Figure 14.

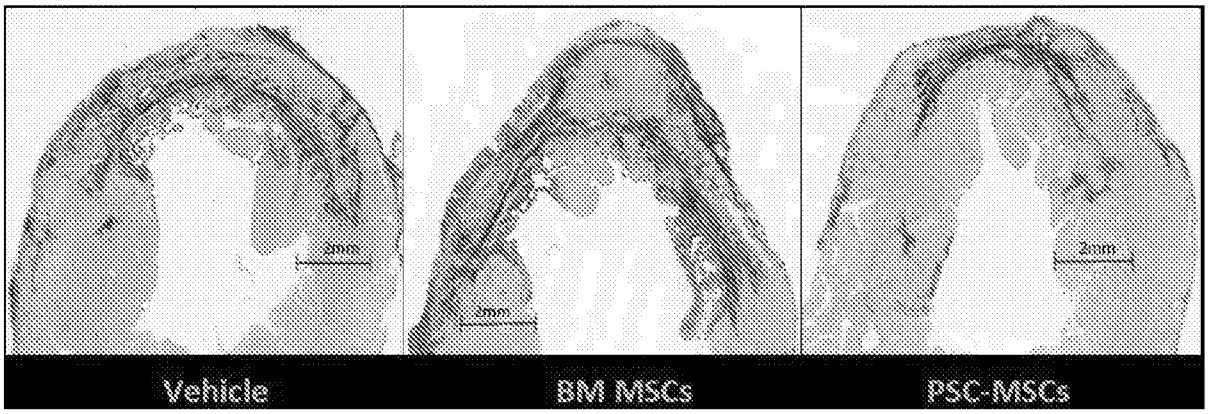


Figure 15.

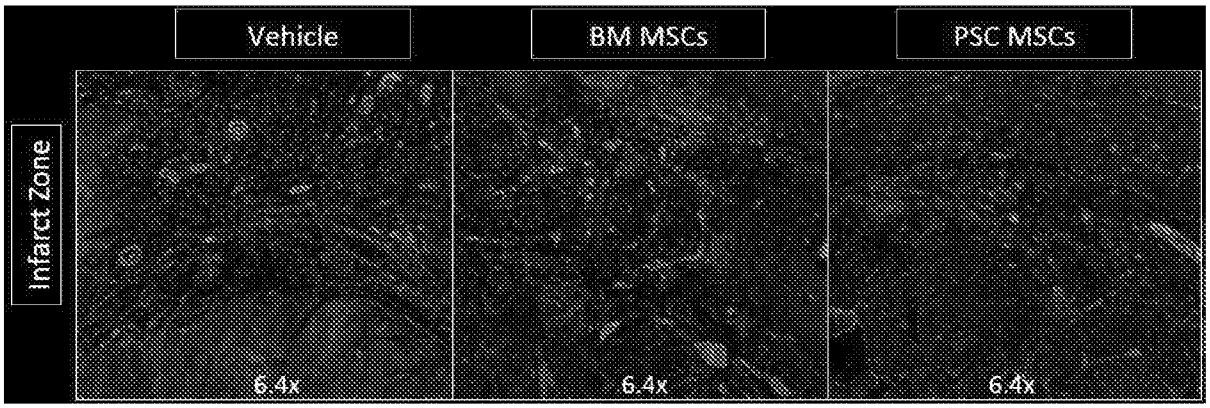


Figure 16.

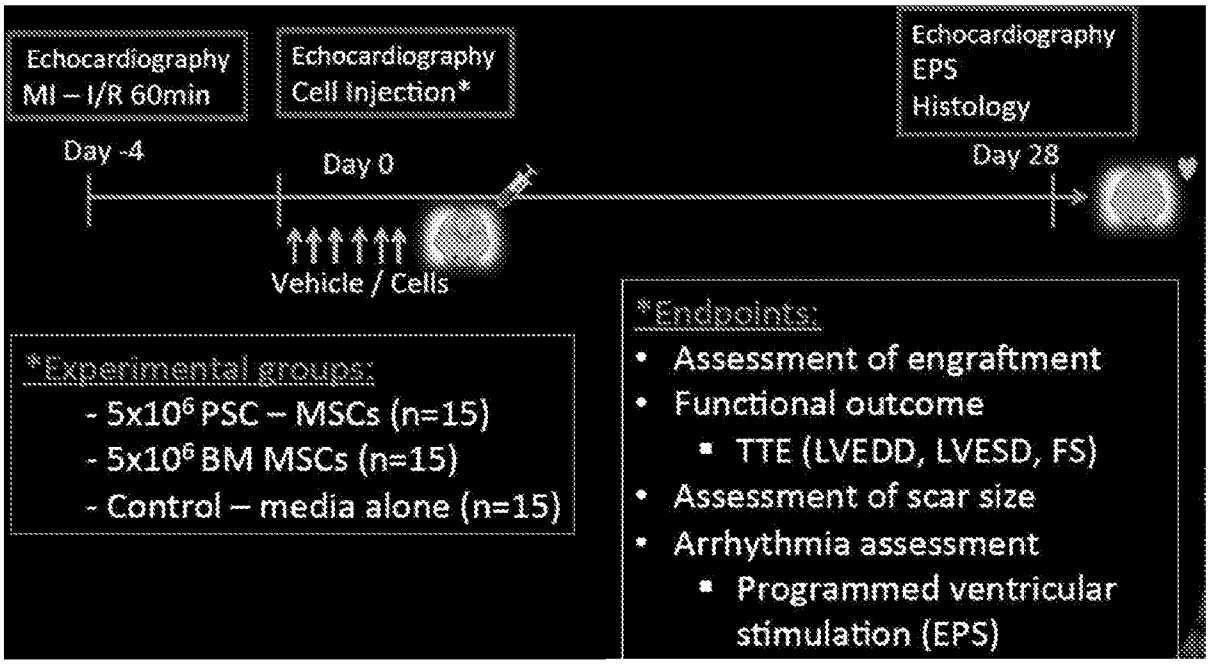


Figure 17.

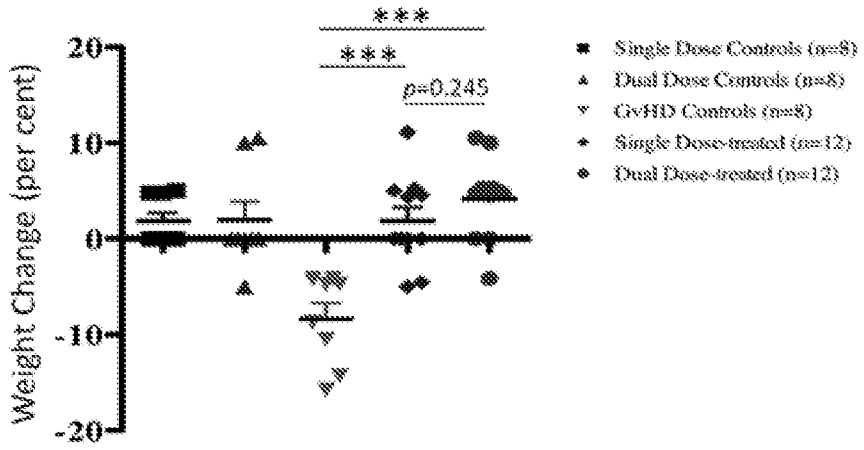


Figure 18.

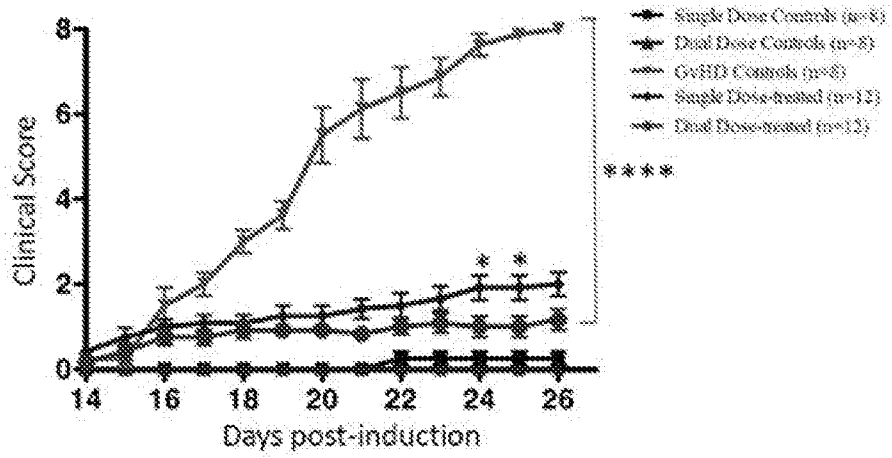
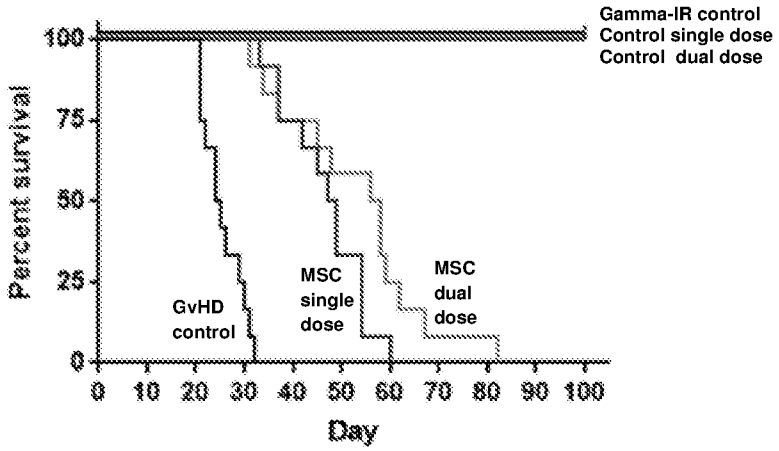


Figure 19.



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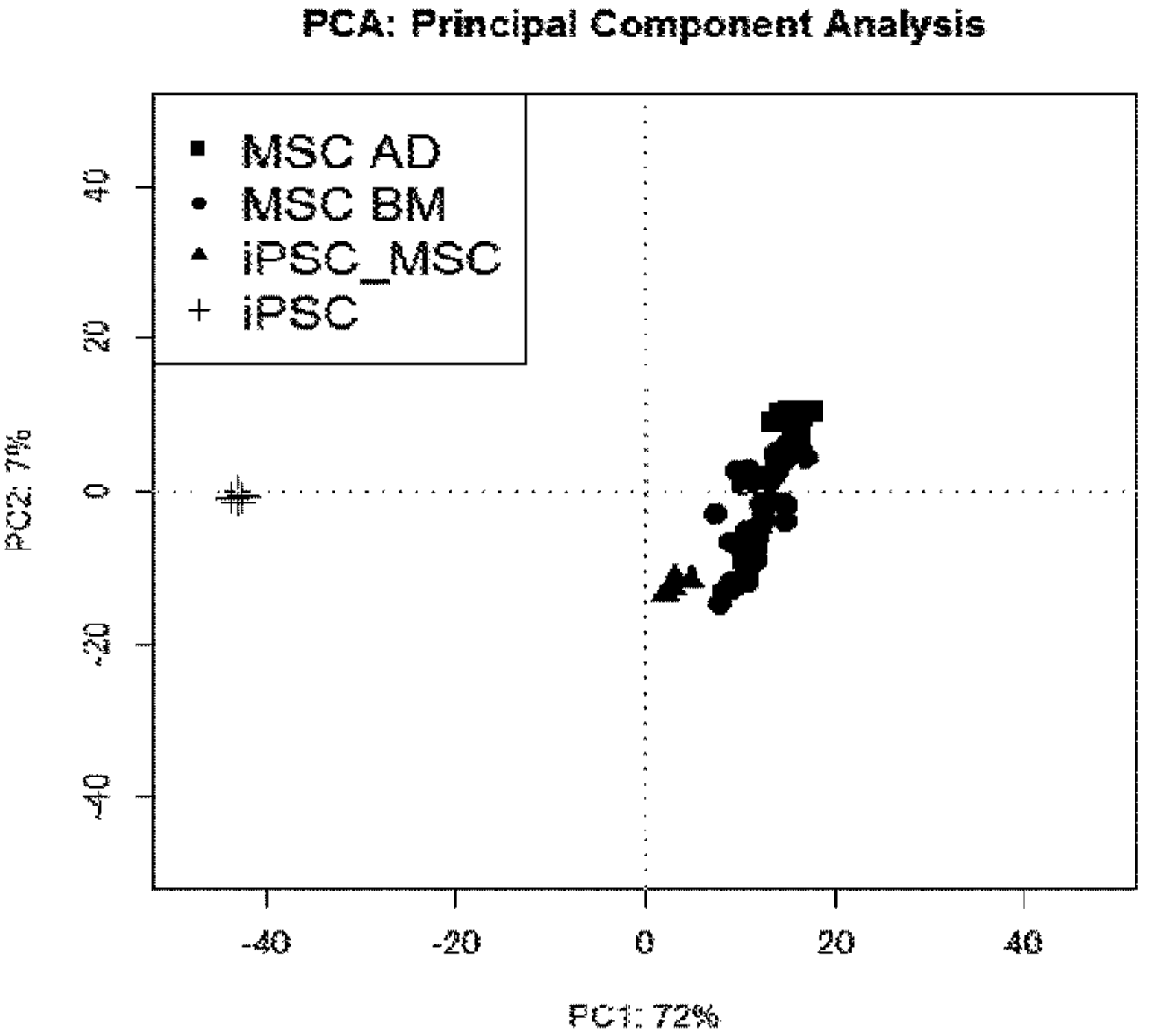
PCA: Principal Component Analysis
MSC AD
MSC BM
iPSC_MSC
iPSC
PC2: 7%
PC1: 72%
40
20
0
-20
-40
-40
-20
0
20
40