

MESENCHYMAL LINEAGE PRECURSOR OR STEM CELLS (MLPSCS) FOR USE IN METHODS OF TREATING CONGENITAL HEART DISEASE

Technical Field

[1] The present disclosure relates to MLPSC mediated methods of treating congenital heart disease. Such methods may be particularly suitable for treating subjects with hypoplastic left heart syndrome.

Background

[2] Hypoplastic left heart syndrome (HLHS) is a spectrum of severe congenital heart disease (CHD) characterized by underdeveloped left-sided cardiac structures. Single ventricle palliation through the Fontan operation is standard-of-care but is associated with long-term morbidity secondary to right heart failure including renal failure, hepatic steatosis, arrhythmias, and protein-losing enteropathy. Attempts at early biventricular conversion (BiV) may carry higher perioperative risk and lead to long-term diastolic dysfunction in some patients. An intermediate option is staged left ventricular recruitment with initial Stage 1 palliation followed by a superior cavopulmonary anastomosis (Glenn) surgery and surgical manoeuvres to gradually promote volume-loading of the hypoplastic left ventricle (LV) including aortic and mitral valvuloplasty, resection of endocardial fibroelastosis (EFE), and atrial septal defect restriction. Following staged recruitment, patients with sufficient LV growth are candidates for BiV or reverse 1.5 ventricle (1.5V) repair. Patients with inadequate LV growth usually progress to a Fontan palliation. These strategies are helpful in some patients, however a majority of patients with borderline LV are still unable to achieve successful biventricular conversion.

[3] Clearly, there is a need in the art for treating congenital heart disease, in particular in the context of HLHS.

Summary

[4] The present inventors have surprisingly identified that mesenchymal lineage precursor or stem cells (MLPSCs) can improve cardiac function, in particular left ventricular end diastolic function (LVEDF) and left ventricular end systolic function (LVESV) in paediatric subjects with congenital heart disease, hypoplastic left heart syndrome (HLHS). This represents a significant advance in the art in a high risk patient population. For example, the inventors' findings indicate improved cardiac performance can be achieved in treated subjects. In certain embodiments, the findings indicate improved prospects for BiV/1.5V conversion in HLHS subjects treated with MLPSCs.

Thus, the findings support embodiments such as the use of MLPSCs as an adjuvant therapy for definitive left ventricular surgical procedures to avoid the need for Fontan surgery.

[5] Accordingly, in an embodiment, the present disclosure relates to a method of treating congenital heart disease in a subject, the method comprising administering to the subject a composition comprising a population of culture expanded mesenchymal lineage precursor or stem cells (MLPSCs) or conditioned media obtained therefrom. In an example, the subject has hypoplastic left heart syndrome.

[6] In an example, the MLPSCs are immune privileged.

[7] In an example, the MLPSCs are allogenic.

[8] In an example, the subject has undergone single ventricle palliation, preferably wherein the subject has undergone a Norwood procedure. In another example, the subject has undergone bidirectional Glenn surgery. In an example, treatment according to the present disclosure comprises a bidirectional Glenn surgery and administering the composition. In an example, the composition is administered at the time of the bidirectional Glenn surgery.

[9] In an example, compositions administered according to the present disclosure comprises 20 million MLPSCs. In an example, the composition is administered to the heart muscle. In an example, the composition is administered to the heart wall of the left ventricle (LV). In an example, the composition is administered to the LV myocardium. In an example, the composition is administered to the LV endocardium. In an example, the composition is administered over 7 – 15 injections to the LV endocardium. In an example, at least two of the injections are administered to the two LV papillary muscles. In an example, the remainder of the injections are administered over the upper, mid, and apical regions of the LV endocardium.

[10] In an example, treatment increases LVEDV and/or LVESV from baseline 12 months after administering the composition. In an example, LVEDV is increased by at least 50 ml/m². In an example, LVEDV is increased by at least 65 ml/m². In an example, LVEDV is increased by at least 80 ml/m². In an example, LVEDV is increased between 60 ml/m² and 120 ml/m². In an example, LVESV is increased by at least 15 ml/m². In another example, LVESV is increased by at least 20 ml/m². In an example, LVESV is increased by at least 25 ml/m². In an example, LVESV is increased between 20 ml/m² and 50 ml/m².

[11] In an example, LVESV/LVEDV is measured by 3-D echocardiography.

[12] In an example, an increase in LVEDV and/or LVESV is determined relative to a subject with a comparable congenital heart disease that has not been administered MLPSCs.

[13] In an example, treatment increases left ventricular end diastolic pressure (LVEDP) from baseline 12 months after administering the composition. In an example, treatment increases LVEDP by at least 5 mmHg. In an example, treatment increases LVEDP by at least 1 mmHg. In an example, treatment increases LVEDP by at least 3 mmHg.

[14] In an example, treatment increases LV stroke volume. In an example, treatment increases LV stroke volume by at least 5 to 20 ml/m².

[15] In an example, the subject has persistent inflammation prior to treatment. In an example, the subject's CRP level is elevated prior to treatment.

[16] In an example, the composition has been culture expanded in a cell culture media comprising at least one pro-inflammatory cytokine. In an example, the media contains serum which comprises the pro-inflammatory cytokine(s). In another example, the media comprises a non-fetal serum. In an example, the serum is a newborn mammalian serum. In an example, the serum is newborn calf serum (NBCS). In an example, the serum is obtained no more than 21 days after birth. In an example, the serum is obtained between the day of birth and 10 days after birth. In an example, the media comprises at least 5% (v/v) newborn calf serum.

[17] In an example, the MLPSCs are mesenchymal precursor cells (MPCs). In an example, the MLPSCs are MPCs that are isolated from bone mononuclear cells with an anti-STRO-3 antibody before culture expansion. In another example, before culture expansion, the MPCs are isolated from bone mononuclear cells via immunoselection to provide a population of cells which comprises STRO-3+ cells.

[18] In another example, the MLPSCs are mesenchymal stem cells (MSCs).

[19] In an example, the MLPSCs have been cryopreserved prior to administration.

[20] In an example, the composition further comprises Plasma-Lyte A, dimethyl sulfoxide (DMSO), human serum albumin (HSA). In an example, the composition comprises greater than 6.68×10^6 viable cells/mL.

[21] In an example, the composition comprises human bone marrow-derived allogeneic MPCs isolated from bone mononuclear cells with anti-STRO-3 antibodies, expanded *ex vivo* in culture media comprising NBCS, and cryopreserved. For example, the MLPSCs can be a population of MLPSCs that have been culture expanded from a population of cells isolated with an anti-STRO-3 antibody. In an example, the MLPSCs in the composition are characterised by one or more of the following:

- the MLPSCs express a level of angiogenin greater than about 1200 pg/ml under culture conditions;

- conditioned media obtained from the MLPSCs under culture conditions induce endothelial network formation greater than about $0.12 \text{ mm}^2/\text{mm}^2$;

- conditioned media obtained from the MLPSCs under culture conditions induce endothelial network length greater than about $5 \text{ mm}^2/\text{mm}^2$;

- [22] - conditioned media obtained from the MLPSCs under culture conditions induce endothelial branch length greater than about $15 \text{ 1}/\text{mm}^2$.

[23] In an example, the present disclosure relates to use of MLPSCs as an adjuvant to a definitive left ventricular surgery in subjects with a congenital heart disease, such as hypoplastic left heart syndrome.

[24] The present disclosure also provides a method of adjuvant therapy comprising administering to a subject a composition comprising a population of culture expanded mesenchymal lineage precursor or stem cells (MLPSCs) or conditioned media obtained therefrom, wherein the subject has a congenital heart disease, and wherein the composition is administered as an adjuvant therapy prior to a definitive left ventricular surgery.

[25] The present disclosure also provides a composition comprising MLPSCs for use as an adjuvant therapy in a subject with congenital heart disease. In an example, the composition is administered prior to a definitive left ventricular surgery.

[26] In an example, the subject has hypoplastic left heart syndrome. In an example, the definitive left ventricular surgery is a bidirectional Glenn surgery. In an example, definitive left ventricular surgery is biventricular conversion. In an example, the definitive left ventricular surgery is performed within 2 years after administering the composition. In an example, the definitive left ventricular surgery is performed at least 12 months after administering the composition. In an example, the definitive left ventricular surgery is performed between 12 months and 2 years after administering the composition.

[27] The present disclosure also provides a method of improving left ventricular function in a subject suffering from congenital heart disease, the method comprising administering to the subject a composition comprising a population of culture expanded mesenchymal lineage precursor or stem cells (MLPSCs) or conditioned media obtained therefrom in an amount effective to increase left ventricular end diastolic volume (LVEDV), left ventricular end systolic volume (LVESV), LV stroke volume, and/or left ventricular end diastolic pressure (LVEDP). In an example, treatment also preserves left

ventricular ejection fraction (LVEF). In an example, LVEF is preserved for at least 12 months after administration of MLPSCs.

[28] The present disclosure also provides a method of treating left ventricular dysfunction in a subject suffering from congenital heart disease, the method comprising administering to the subject a composition comprising a population of culture expanded mesenchymal lineage precursor or stem cells (MLPSCs) or conditioned media obtained therefrom in an amount effective to increase left ventricular end diastolic volume (LVEDV), left ventricular end systolic volume (LVESV), LV stroke volume, and/or left ventricular end diastolic pressure (LVEDP).). In an example, treatment also preserves left ventricular ejection fraction (LVEF). In an example, LVEF is preserved for at least 12 months after administration of MLPSCs.

[29] In an example, LVEDV, LVESV, LV stroke volume, and/or LVEDP is increased from baseline 12 months after administering the composition. In an example, LVEDV is increased by at least 50 ml/m². In an example, LVEDV is increased by at least 65 ml/m². In an example, LVEDV is increased by at least 80 ml/m². In an example, LVEDV is increased by between 60 ml/m² and 120 ml/m². In an example, LVESV is increased by at least 15 ml/m². In an example, LVESV is increased by at least 20 ml/m². In an example, LVESV is increased by at least 25 ml/m². In an example, LVESV is increased by at least between 20 ml/m² and 50 ml/m². In an example, LV stroke volume is increased by at least 5 to 20 ml/m². In an example, LVEDP is increased by at least 5 mmHg.

[30] In an example, MLPSCs administered according to the disclosure are culture expanded from a population of cells which comprise about 0.1% to 75% STRO-1+ cells. In an example, the population of cells which comprise about 0.1% to 75% STRO-1+ cells are isolated using a STRO-3 antibody.

Brief Description of Drawings

[31] **Figure 1:** Time from LV recruitment surgery to BiV or 1.5V conversion surgery within two years of pre-planned follow-up, by treatment assigned.

[32] **Figure 2:** Change in LV end diastolic volume (A), LV end systolic volume (B), and LV mass (C) from baseline to primary efficacy analysis at one year, by treatment assigned: 3-D echocardiography.

[33] **Figure 3:** Relationship between LVEDP and cMRI LV stroke volume (mL) at baseline and month 12 by treatment.

Detailed DescriptionGeneral Techniques and Definitions

[34] Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular biology, stem cell culture, immunology, and biochemistry).

[35] Unless otherwise indicated, cell culture techniques and assays utilized in the present disclosure are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), and F.M. Ausubel et al. (editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present), Ed Harlow and David Lane (editors) Antibodies: A Laboratory Manual, Cold Spring Harbour Laboratory, (1988), and J.E. Coligan et al. (editors) Current Protocols in Immunology, John Wiley & Sons (including all updates until present).

[36] The term “and/or”, e.g., “X and/or Y” shall be understood to mean either “X and Y” or “X or Y” and shall be taken to provide explicit support for both meanings or for either meaning.

[37] As used herein, the term “about”, unless stated to the contrary, refers to +/- 10%, more preferably +/- 5%, of the designated value.

[38] In an example, a sample is obtained from a patient or subject (e.g. a blood sample) and the level of a substance is measured in the sample. For example, a blood sample can be obtained to measure the level of CRP in a subject.

[39] "C-reactive protein" or "CRP" is an inflammatory mediator. CRP levels are raised under conditions of acute inflammatory recurrence and rapidly normalize once the inflammation subsides. Accordingly, CRP is an effective marker of persistent inflammation. In an example, subjects treated according to the present disclosure may have elevated CRP. The term “elevated CRP” is used in the context of the present disclosure to refer to CRP levels that are increased relative to baseline CRP levels. In an example, CRP levels ≥ 1 mg/L are elevated. In another example, CRP levels ≥ 1.5 mg/L are elevated. In another example, CRP levels ≥ 2 mg/L are elevated. In an example, persistent inflammation is characterised by CRP levels ≥ 2 mg/L.

[40] The term “level” is used to define the amount of a particular substance present in a sample, cell culture medium, serum preparation or compositions of the present disclosure. For example, a particular concentration (e.g. mg/L), weight, percentage (e.g. v/v%) or ratio can be used to define the level of a particular substance.

[41] The term “conditioned media” is used in the context of the present disclosure to refer to media obtained from MLPSCs under culture conditions. Such media contains the MLPSC secretome, proteins shed from the surface of MLPSCs and, other particles such as extracellular vesicles. In certain embodiments such as those relating to culture of MLPSCs in serum containing one or more pro-inflammatory cytokines, conditioned media of the disclosure contains pro-angiogenic factors such as extracellular vesicles, Angiogenin or secreted metabolites such as prostaglandin E2. The pro-angiogenic capabilities of conditioned media disclosed herein and/or factors obtained therefrom can be confirmed, if necessary, using one or more of the angiogenesis assays disclosed herein (e.g. endothelial network formation; endothelial length; endothelial branch length). In certain examples, the present disclosure relates to extracellular vesicles such as exosomes that have been obtained from conditioned media obtained from MLPSCs under culture conditions. In an example, the conditioned media is obtained when the MLPSCs are in exponential growth phase. In an example, the conditioned media is obtained after at least two or three days in culture. In another example, the conditioned media is obtained after about 30 to 84 hours of culture.

[42] In an example, the level of a particular marker in an MLPSC population of the disclosure or conditioned media obtained from the same, such as a pro-angiogenic factor(s), is determined under culture conditions. The term “culture conditions” is used to refer to cells growing in culture. In an example, culture conditions refers to an actively dividing population of cells. Such cells may, in an example, be in exponential growth phase. Alternatively, such cells may be in a stationary phase.

[43] In an example, the level is expressed in terms of how much of a particular marker is released from cells described herein under culture conditions. In an example, the level of a particular marker is measured in a population of cells (or supernatant obtained following cell culture of the same) and divided by the number of cells in the population. In this example, the level may be presented in units (e.g. pg) per 10^6 cells.

[44] In an example, the level is expressed in mg/L. For example, a level of CRP can be expressed in mg/L. In an example, the level is expressed in ng/ml. For example, a level of VEGF can be expressed in ng/ml. In an example, a level of SDF-1 α can be expressed in ng/ml. In an example, the level is expressed in pg/ml. For example, a level

of NT-proBNP can be expressed in pg/ml. In an example, a level of angiogenin can be expressed in pg/ml.

[45] In an example, the level of a particular marker can be determined by taking a sample of cell culture media and measuring the level of marker in the sample. In another example, the level of a particular marker can be determined by taking a sample of cells and measuring the level of the marker in the cell lysate. Those of skill in the art will appreciate that secreted markers can be measured by sampling the culture media while markers expressed on the surface of the cell may be measured by assessing a sample of cell lysate. In an example, the sample is taken when the cells are in exponential growth phase. In an example, the sample is taken after at least two or three days in culture. In another example, the sample is taken after about 30 to 84 hours of culture. In another example, the sample is taken when the cells are in a stationary phase.

[46] In another example, the level of a particular marker is determined using fluorescence-activated cell sorting (FACS) using appropriate antibodies such as anti-CD25. Further antibodies may also be employed if required to distinguish CD25+ cell types. It will be appreciated that similar methods may also be used to determine the level of other markers disclosed herein such as angiogenin. In these examples, co-culture may not be required to determine the level. For example, the level of angiogenin may be measured in a population of MLPSCs under culture conditions.

[47] In another example, the level is measured based on an assessment of conditioned media (or properties thereof) obtained from a population of MLPSCs under culture conditions. For example, conditioned media can be obtained from a population of MLPSCs disclosed herein under culture conditions before being used in one or more angiogenesis assays disclosed below.

[48] Culture expanding cells from a cryopreserved intermediate means thawing cells subject to cryogenic freezing and *in vitro* culturing under conditions suitable for growth of the cells.

[49] As used herein, the terms “treating”, “treat”, “treatment”, “reducing progression” include administering a population of mesenchymal lineage stem or precursor cells cultured according to the present disclosure and/or progeny thereof and/or soluble factors derived therefrom and/or extracellular vesicles derived therefrom to thereby reduce or eliminate at least one symptom of congenital heart disease or, in the context of reducing progression, delay development of the same. In some examples, treatment improves or increases at least one symptom or parameter in a subject suffering from congenital heart disease, such as left ventricular function.

[50] In an example, the present disclosure encompasses selecting certain subjects with congenital heart disease for treatment with a cellular composition disclosed herein. In an example, subjects that have undergone a single ventricle palliation are selected for treatment. In an example, subjects with hypoplastic left heart syndrome are selected for treatment. In an example, subjects with persistent inflammation are selected for treatment. In an example, persistent inflammation is determined based on CRP level. For example, subjects with persistent inflammation have elevated CRP. In an example, subjects with CRP levels ≥ 2 mg/L are selected for treatment. In an example, persistent inflammation is determined based on IL-6 level. For example, subjects with persistent inflammation have elevated IL-6. In an example, subjects with persistently elevated IL-6 levels post-single ventricle palliation are selected for treatment.

[51] The term “subject” as used herein refers to a human subject. In an example, the subject is a paediatric subject. For example, the subject can be an infant. Terms such as “subject”, “patient” or “individual” are terms that can, in context, be used interchangeably in the present disclosure.

[52] In an example, compositions of the disclosure comprise genetically unmodified MLPSCs. As used herein, the term “genetically unmodified” refers to cells that have not been modified by transfection with a nucleic acid. For the avoidance of doubt, in the context of the present disclosure a MLPSC transfected with a nucleic acid encoding a protein would be considered genetically modified.

[53] The term “angiogenic marker” as used herein refers an indicator of angiogenesis. As used herein, “angiogenic markers” include pro-angiogenic molecules, for example, VEGF, angiogenin, and SDF-1 α . In another example, angiogenic markers are cellular indicators of angiogenesis, for example, endothelial network formation, endothelial network length, and endothelial branch length. In this example, cellular indicators of angiogenesis are determined in an in-vitro angiogenesis assay as disclosed herein. In an example, angiogenic marker characterisation may be used to characterise a MLPSC population disclosed herein.

[54] As used herein, the term “sample” refers to an extract from a subject or cell culture in which the level of a particular marker can be measured. The “sample” includes extracts and/or derivatives and/or fractions of the sample. In an example, the sample is an extract from a subject in which CRP levels can be measured. In the present disclosure, any biological material can be used as the above-mentioned sample so long as it can be collected from the subject or cell culture and assayed to determine the level of a marker disclosed herein (e.g. level of CRP in a subject). In an example, the sample is a blood

sample. For example, the blood sample can be obtained from a subject with hypoplastic left heart syndrome.

[55] In an example, the “sample” is a population of cells, for example a population of cells under culture conditions. In an example, the sample is supernatant obtained following cell culture, for example, cell conditioned media. In these examples, the sample is any extract of cell culture in which certain markers (e.g. angiogenic) can be measured. In an example, the sample is contacted with another cell population to determine the level of an angiogenic marker.

[56] In an example, the present disclosure encompasses selecting a population of culture expanded MLPSCs of a certain potency for use in methods of treatment disclosed herein. The term “potency” as used herein refers to the specific ability or capacity of the MLPSCs to effect a given result. In an example, the result is a therapeutic result, for example an improvement in cardiac outcomes as disclosed herein.

[57] “Therapeutic efficacy” is used in the context of the present disclosure to refer to MLPSCs and compositions disclosed herein that can treat, inhibit and/or prevent disease. For example, therapeutically effective MLPSCs and compositions disclosed herein can treat, inhibit and/or prevent congenital heart disease such as hypoplastic left heart syndrome or improve left ventricular function in these subjects. For example, therapeutically effective MLPSCs and compositions disclosed herein can increase one or parameters such as LVEDV, LVESV and/or LVEF in subjects with congenital heart disease such as hypoplastic left heart syndrome.

[58] “Biological activity” is used in the context of the present disclosure to define MLPSCs and compositions disclosed herein based on a particular activity. In an example, the biological activity is pro-angiogenic and/or anti-inflammatory activity. In an example, the biological activity is capacity to increase in-vitro angiogenesis. In an example, the biological activity is the increased expression of one or more angiogenic markers. In an example, the biological activity is angiogenic potential, as determined by the level of one or more angiogenic markers. In an example, biological activity is characterised by an improved clinical outcome(s) (e.g. survival) and/or parameter(s) (e.g. LVEDV and/or LVESV).

[59] The term "clinically proven" (used independently or to modify the term "effective") shall mean that efficacy has been proven by a clinical trial wherein the clinical trial has met the approval standards of U.S. Food and Drug Administration, EMEA or a corresponding national regulatory agency. For example, the clinical study may be an adequately sized, randomized, double-blinded study used to clinically prove the effects of the composition. In an example, a clinically proven effective amount is an

amount shown by a clinical trial to meet a specified endpoint. In an example, the end point is improved cardiac function as determined based on one or parameters such as LVEDV, LVESV and/or LVEF.

[60] Accordingly, the terms "clinically proven efficacy" and "clinically proven effective" can be used in the context of the present disclosure to refer to a dose, dosage regimen, treatment or method disclosed herein. Efficacy can be measured based on change in the course of the disease in response to administering a composition disclosed herein. For example, a composition of the disclosure is administered to a subject in an amount and for a time sufficient to induce an improvement, preferably a sustained improvement, in at least one indicator that reflects the severity of cardiovascular disease. Various indicators that reflect the severity of the disease can be assessed for determining whether the amount and time of the treatment is sufficient. Such indicators include, for example, clinically recognized indicators of disease severity or symptoms. In an example, the degree of improvement is determined by a physician, who can make this determination based on signs, symptoms, or other test results (e.g. 3-D echocardiography; LVEF; LVESV). In an example, a clinically proven effective amount improves patient survival. In another example, a clinically proven effective amount reduces a subjects risk of mortality. In another example, a clinically proven effective amount increases 100 day survival. In another example, a clinically proven effective amount increases LVEDV and/or LVESV. In another example, a clinically proven effective amount increases prospects for biventricular conversion surgery. In an example, methods of the disclosure administer a clinically proven effective amount of a composition disclosed herein.

[61] Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

[62] Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or group of compositions of matter.

[63] Those skilled in the art will appreciate that the disclosure described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the disclosure includes all such variations and modifications. The disclosure also includes all of the steps, features, compositions and compounds referred

to or indicated in this specification, individually or collectively, and any and all combinations or any two or more of said steps or features.

[64] The present disclosure is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the disclosure, as described herein.

[65] Any example disclosed herein shall be taken to apply mutatis mutandis to any other example unless specifically stated otherwise.

Mesenchymal lineage precursor or stem cells (MLPSCs)

[66] As used herein, the term “mesenchymal lineage precursor or stem cell (MLPSC)” refers to undifferentiated multipotent cells that have the capacity to self-renew while maintaining multipotency and the capacity to differentiate into a number of cell types either of mesenchymal origin, for example, osteoblasts, chondrocytes, adipocytes, stromal cells, fibroblasts and tendons, or non-mesodermal origin, for example, hepatocytes, neural cells and epithelial cells. For the avoidance of doubt, a “mesenchymal lineage precursor cell” refers to a cell which can differentiate into a mesenchymal cell such as bone, cartilage, muscle and fat cells, and fibrous connective tissue.

[67] The term "mesenchymal lineage precursor or stem cells" includes both parent cells and their undifferentiated progeny. The term also includes mesenchymal precursor cells, multipotent stromal cells, mesenchymal stem cells (MSCs), perivascular mesenchymal precursor cells, and their undifferentiated progeny.

[68] Mesenchymal lineage precursor or stem cells can be autologous, allogeneic, xenogenic, syngenic or isogenic. Autologous cells are isolated from the same individual to which they will be reimplanted. Allogeneic cells are isolated from a donor of the same species. Xenogenic cells are isolated from a donor of another species. Syngenic or isogenic cells are isolated from genetically identical organisms, such as twins, clones, or highly inbred research animal models.

[69] In an example, the mesenchymal lineage precursor or stem cells are allogeneic. In an example, the allogeneic mesenchymal lineage precursor or stem cells are culture expanded and cryopreserved.

[70] In an example, the MLPSCs are allogenic. In an example, the MLPSCs are immune privileged.

[71] Mesenchymal lineage precursor or stem cells reside primarily in the bone marrow, but have also shown to be present in diverse host tissues including, for example,

cord blood and umbilical cord, adult peripheral blood, adipose tissue, trabecular bone and dental pulp. They are also found in skin, spleen, pancreas, brain, kidney, liver, heart, retina, brain, hair follicles, intestine, lung, lymph node, thymus, ligament, tendon, skeletal muscle, dermis, and periosteum; and are capable of differentiating into germ lines such as mesoderm and/or endoderm and/or ectoderm. Thus, mesenchymal lineage precursor or stem cells are capable of differentiating into a large number of cell types including, but not limited to, adipose, osseous, cartilaginous, elastic, muscular, and fibrous connective tissues. The specific lineage-commitment and differentiation pathway which these cells enter depends upon various influences from mechanical influences and/or endogenous bioactive factors, such as growth factors, cytokines, and/or local microenvironmental conditions established by host tissues.

[72] The terms “enriched”, “enrichment” or variations thereof are used herein to describe a population of cells in which the proportion of one particular cell type or the proportion of a number of particular cell types is increased when compared with an untreated population of the cells (e.g., cells in their native environment). In one example, a population enriched for mesenchymal lineage precursor or stem cells comprises at least about 0.1% or 0.5% or 1% or 2% or 5% or 10% or 15% or 20% or 25% or 30% or 50% or 75% mesenchymal lineage precursor or stem cells. In this regard, the term “population of cells enriched for mesenchymal lineage precursor or stem cells” will be taken to provide explicit support for the term “population of cells comprising X% mesenchymal lineage precursor or stem cells”, wherein X% is a percentage as recited herein. The mesenchymal lineage precursor or stem cells can, in some examples, form clonogenic colonies, e.g. CFU-F (fibroblasts) or a subset thereof (e.g., 50% or 60% or 70% or 70% or 90% or 95%) can have this activity.

[73] In an example of the present disclosure, the mesenchymal lineage precursor or stem cells are mesenchymal stem cells (MSCs). The MSCs may be a homogeneous composition or may be a mixed cell population enriched in MSCs. Homogeneous MSC compositions may be obtained by culturing adherent marrow or periosteal cells, and the MSCs may be identified by specific cell surface markers which are identified with unique monoclonal antibodies. A method for obtaining a cell population enriched in MSCs is described, for example, in U.S. Patent No. 5,486,359. Alternative sources for MSCs include, but are not limited to, blood, skin, cord blood, muscle, fat, bone, and perichondrium. In an example, the MSCs are allogeneic. In an example, the MSCs are cryopreserved. In an example, the MSCs are culture expanded and cryopreserved.

[74] In another example, the mesenchymal lineage precursor or stem cells are CD29+, CD54+, CD73+, CD90+, CD102+, CD105+, CD106+, CD166+, MHC1+ MSCs.

[75] Isolated or enriched mesenchymal lineage precursor or stem cells can be expanded *in vitro* by culture. Isolated or enriched mesenchymal lineage precursor or stem cells can be cryopreserved, thawed and subsequently expanded *in vitro* by culture.

[76] In one example, isolated or enriched mesenchymal lineage precursor or stem cells are seeded at 50,000 viable cells/cm² in culture medium (serum free or serum-supplemented), for example, alpha minimum essential media (α MEM) supplemented with 5% fetal bovine serum (FBS) and glutamine, and allowed to adhere to the culture vessel overnight at 37°C, 20% O₂. As used herein, the terms “culture media” and “culture medium” are used interchangeably. The culture medium is subsequently replaced and/or altered as required and the cells cultured for a further 68 to 72 hours at 37°C, 5% O₂.

[77] As will be appreciated by those of skill in the art, cultured mesenchymal lineage precursor or stem cells are phenotypically different to cells *in vivo*. For example, in one embodiment they express one or more of the following markers, CD44, NG2, DC146 and CD140b. Cultured mesenchymal lineage precursor or stem cells are also biologically different to cells *in vivo*, having a higher rate of proliferation compared to the largely non-cycling (quiescent) cells *in vivo*.

[78] In one example, the population of cells is enriched from a cell preparation comprising STRO-1+ cells in a selectable form. In this regard, the term “selectable form” will be understood to mean that the cells express a marker (e.g., a cell surface marker) permitting selection of the STRO-1+ cells. The marker can be STRO-1, but need not be. For example, as described and/or exemplified herein, cells (e.g., mesenchymal precursor cells) expressing STRO-2 and/or STRO-3 (TNAP) and/or STRO-4 and/or VCAM-1 and/or CD146 and/or 3G5 also express STRO-1 (and can be STRO-1bright). Accordingly, an indication that cells are STRO-1+ does not mean that the cells are selected solely by STRO-1 expression. In one example, the cells are selected based on at least STRO-3 expression, e.g., they are STRO-3+ (TNAP+). For example, the MPCs can be isolated from bone mononuclear cells with an anti-STRO-3 antibody.

[79] Reference to selection of a cell or population thereof does not necessarily require selection from a specific tissue source. As described herein STRO-1+ cells can be selected from or isolated from or enriched from a large variety of sources. That said, in some examples, these terms provide support for selection from any tissue comprising STRO-1+ cells (e.g., mesenchymal precursor cells) or vascularized tissue or tissue

comprising pericytes (e.g., STRO-1+ pericytes) or any one or more of the tissues recited herein.

[80] In one example, the cells used in the present disclosure express one or more markers individually or collectively selected from the group consisting of TNAP+, VCAM-1+, THY-1+, STRO-2+, STRO-4+ (HSP-90 β), CD45+, CD146+, 3G5+ or any combination thereof.

[81] By "individually" is meant that the disclosure encompasses the recited markers or groups of markers separately, and that, notwithstanding that individual markers or groups of markers may not be separately listed herein the accompanying claims may define such marker or groups of markers separately and divisibly from each other.

[82] By "collectively" is meant that the disclosure encompasses any number or combination of the recited markers or groups of markers, and that, notwithstanding that such numbers or combinations of markers or groups of markers may not be specifically listed herein the accompanying claims may define such combinations or sub-combinations separately and divisibly from any other combination of markers or groups of markers.

[83] As used herein the term "TNAP" is intended to encompass all isoforms of tissue non-specific alkaline phosphatase. For example, the term encompasses the liver isoform (LAP), the bone isoform (BAP) and the kidney isoform (KAP). In one example, the TNAP is BAP. In one example, TNAP as used herein refers to a molecule which can bind the STRO-3 antibody produced by the hybridoma cell line deposited with ATCC on 19 December 2005 under the provisions of the Budapest Treaty under deposit accession number PTA-7282.

[84] Furthermore, in one example, the STRO-1+ cells are capable of giving rise to clonogenic CFU-F.

[85] In one example, a significant proportion of the STRO-1+ cells are capable of differentiation into at least two different germ lines. Non-limiting examples of the lineages to which the STRO-1+ cells may be committed include bone precursor cells; hepatocyte progenitors, which are multipotent for bile duct epithelial cells and hepatocytes; neural restricted cells, which can generate glial cell precursors that progress to oligodendrocytes and astrocytes; neuronal precursors that progress to neurons; precursors for cardiac muscle and cardiomyocytes, glucose-responsive insulin secreting pancreatic beta cell lines. Other lineages include, but are not limited to, odontoblasts, dentin-producing cells and chondrocytes, and precursor cells of the following: retinal pigment epithelial cells, fibroblasts, skin cells such as keratinocytes, dendritic cells, hair follicle cells, renal duct epithelial cells, smooth and skeletal muscle cells, testicular

progenitors, vascular endothelial cells, tendon, ligament, cartilage, adipocyte, fibroblast, marrow stroma, cardiac muscle, smooth muscle, skeletal muscle, pericyte, vascular, epithelial, glial, neuronal, astrocyte and oligodendrocyte cells.

[86] In an example, mesenchymal lineage precursor or stem cells are obtained from a single donor, or multiple donors where the donor samples or mesenchymal lineage precursor or stem cells are subsequently pooled and then culture expanded.

[87] Mesenchymal lineage precursor or stem cells encompassed by the present disclosure may also be cryopreserved prior to administration to a subject. In an example, mesenchymal lineage precursor or stem cells are culture expanded and cryopreserved prior to administration to a subject.

[88] In an example, the present disclosure encompasses mesenchymal lineage precursor or stem cells as well as progeny thereof, soluble factors derived therefrom, and/or extracellular vesicles isolated therefrom. In another example, the present disclosure encompasses mesenchymal lineage precursor or stem cells as well as conditioned medium obtained therefrom under culture conditions. In another example, the present disclosure encompasses mesenchymal lineage precursor or stem cells as well as extracellular vesicles isolated therefrom. For example, it is possible to culture expand mesenchymal precursor lineage or stem cells of the disclosure for a period of time and under conditions suitable for secretion of extracellular vesicles into the cell culture medium. Secreted extracellular vesicles can subsequently be obtained from the culture medium for use in therapy. Such extracellular vesicles can be characterised, if necessary, using one or more of the angiogenesis assays disclosed herein (e.g. endothelial network formation; endothelial length; endothelial branch length).

[89] The term “extracellular vesicles” as used herein, refers to lipid particles naturally released from cells and ranging in size from about 30 nm to as large as 10 microns, although typically they are less than 200 nm in size. They can contain proteins, nucleic acids, lipids, metabolites, or organelles from the releasing cells (e.g., mesenchymal stem cells; STRO-1⁺ cells).

[90] The term “exosomes” as used herein, refers to a type of extracellular vesicle generally ranging in size from about 30 nm to about 150 nm and originating in the endosomal compartment of mammalian cells from which they are trafficked to the cell membrane and released. They may contain nucleic acids (e.g., RNA; microRNAs), proteins, lipids, and metabolites and function in intercellular communication by being secreted from one cell and taken up by other cells to deliver their cargo.

[91] The term “pre-licensing” or “licensing”, as used herein, refers to a process by which MLPSCs achieve functional maturation, whereby, the pre-licensed or licensed

MLPSCs reduce release of inflammatory cytokines when the MLPSCs are administered to a subject to a greater extent than MLPSCs that have not been pre-licensed.

[92] The terms “enriched”, “enrichment” or variations thereof are used herein to describe a population of cells in which the proportion of one particular cell type or the proportion of a number of particular cell types is increased when compared with an untreated population of the cells (*e.g.*, cells in their native environment). In one example, a population enriched for STRO-1+ cells comprises at least about 0.1% or 0.5% or 1% or 2% or 5% or 10% or 15% or 20% or 25% or 30% or 50% or 75% STRO-1+ cells. In this regard, the term “population of cells enriched for STRO-1+ cells” will be taken to provide explicit support for the term “population of cells comprising X% STRO-1+ cells”, wherein X% is a percentage as recited herein. The STRO-1+ cells can, in some examples, form clonogenic colonies, *e.g.* CFU-F (fibroblasts) or a subset thereof (*e.g.*, 50% or 60% or 70% or 80% or 90% or 95%) can have this activity.

[93] In one example, the population of cells is enriched from a cell preparation comprising STRO-1+ cells in a selectable form. In this regard, the term “selectable form” will be understood to mean that the cells express a marker (*e.g.*, a cell surface marker) permitting selection of the STRO-1+ cells. The marker can be STRO-1, but need not be. For example, cells (*e.g.*, mesenchymal precursor cells) expressing STRO-2 and/or STRO-3 (TNAP) and/or STRO-4 and/or VCAM-1 and/or CD146 and/or 3G5 also express STRO-1 (and can be STRO-1^{bright}). Accordingly, an indication that cells are STRO-1+ does not mean that the cells are selected by STRO-1 expression. In one example, the cells are selected based on at least STRO-3 expression, *e.g.*, they are STRO-3+ (TNAP+).

[94] In an example, MLPSCs of the disclosure are culture expanded from a population of MLPSCs that are STRO-1+. In an example, these cells may be isolated using a STRO-3 antibody. In an example, the MLPSCs are culture expanded from a population of MLPSCs which comprise about 0.1% to 75% STRO-1+ cells. In another example, the MLPSCs are culture expanded from a population of MLPSCs which comprise about 0.5% to 75% STRO-1+ cells. In another example, the MLPSCs are culture expanded from a population of MLPSCs which comprise about 0.1% to 75% STRO-1+ cells. In another example, the MLPSCs are culture expanded from a population of MLPSCs which comprise about 0.1% to 75% STRO-1+ cells. In another example, the MLPSCs are culture expanded from a population of MLPSCs which comprise about 1% to 75% STRO-1+ cells. In another example, the MLPSCs are culture expanded from a population of MLPSCs which comprise about 0.1% to 75% STRO-1+

cells. In another example, the MLPSCs are culture expanded from a population of MLPSCs which comprise about 10% to 75% STRO-1+ cells.

[95] Reference to selection of a cell or population thereof does not necessarily require selection from a specific tissue source. As described herein STRO-1+ cells can be selected from or isolated from or enriched from a large variety of sources. That said, in some examples, these terms provide support for selection from any tissue comprising STRO-1+ cells (*e.g.*, mesenchymal precursor cells) or vascularized tissue or tissue comprising pericytes (*e.g.*, STRO-1+ pericytes) or any one or more of the tissues recited herein.

[96] In one example, the mesenchymal lineage precursor or stem cells used in the present disclosure express one or more markers individually or collectively selected from the group consisting of TNAP+, VCAM-1+, THY-1+, STRO-2+, STRO-4+ (HSP-90β), CD45+, CD146+, 3G5+ or any combination thereof.

[97] By use of the term "individually" it is meant that the disclosure encompasses the recited markers or groups of markers separately, and that, notwithstanding that individual markers or groups of markers may not be separately listed herein the accompanying claims may define such marker or groups of markers separately and divisibly from each other.

[98] By use of the term "collectively" it is meant that the disclosure encompasses any number or combination of the recited markers or groups of markers, and that, notwithstanding that such numbers or combinations of markers or groups of markers may not be specifically listed herein the accompanying claims may define such combinations or sub-combinations separately and divisibly from any other combination of markers or groups of markers.

[99] In one example, the STRO-1+ cells are STRO-1^{bright} (syn. STRO-1^{bri}). In another example, the STRO-1^{bri} cells are preferentially enriched relative to STRO-1^{dim} or STRO-1^{intermediate} cells. In another example, the STRO-1^{bri} cells are additionally one or more of TNAP+, VCAM-1+, THY-1+, STRO-2+, STRO-4+ (HSP-90β) and/or CD146+. For example, the cells are selected for one or more of the foregoing markers and/or shown to express one or more of the foregoing markers. In this regard, a cell shown to express a marker need not be specifically tested, rather previously enriched or isolated cells can be tested and subsequently used, isolated or enriched cells can be reasonably assumed to also express the same marker.

[100] In one example, the mesenchymal precursor cells are perivascular mesenchymal precursor cells as defined in WO 2004/85630, characterized by the presence of the perivascular marker 3G5.

[101] A cell that is referred to as being "positive" for a given marker may express either a low (lo or dim) or a high (bright, bri) level of that marker depending on the degree to which the marker is present on the cell surface, where the terms relate to intensity of fluorescence or other marker used in the sorting process of the cells. The distinction of lo (or dim or dull) and bri will be understood in the context of the marker used on a particular cell population being sorted. A cell that is referred to as being "negative" for a given marker is not necessarily completely absent from that cell. This term means that the marker is expressed at a relatively very low level by that cell, and that it generates a very low signal when detectably labelled or is undetectable above background levels, *e.g.*, levels detected using an isotype control antibody.

[102] The term "bright" or "bri" as used herein, refers to a marker on a cell surface that generates a relatively high signal when detectably labelled. Whilst not wishing to be limited by theory, it is proposed that "bright" cells express more of the target marker protein (for example the antigen recognized by STRO-1) than other cells in the sample. For instance, STRO-1^{bri} cells produce a greater fluorescent signal, when labelled with a FITC-conjugated STRO-1 antibody as determined by fluorescence activated cell sorting (FACS) analysis, than non-bright cells (STRO-1^{dull/dim}). In one example, "bright" cells constitute at least about 0.1% of the most brightly labelled bone marrow mononuclear cells contained in the starting sample. In other examples, "bright" cells constitute at least about 0.5%, at least about 1%, at least about 1.5%, or at least about 2%, of the most brightly labelled bone marrow mononuclear cells contained in the starting sample. In an example, STRO-1^{bright} cells have 2 log magnitude higher expression of STRO-1 surface expression relative to "background", namely cells that are STRO-1⁻. By comparison, STRO-1^{dim} and/or STRO-1^{intermediate} cells have less than 2 log magnitude higher expression of STRO-1 surface expression, typically about 1 log or less than "background".

[103] As used herein the term "TNAP" is intended to encompass all isoforms of tissue non-specific alkaline phosphatase. For example, the term encompasses the liver isoform (LAP), the bone isoform (BAP) and the kidney isoform (KAP). In one example, the TNAP is BAP. In one example, TNAP as used herein refers to a molecule which can bind the STRO-3 antibody produced by the hybridoma cell line deposited with ATCC on 19 December 2005 under the provisions of the Budapest Treaty under deposit accession number PTA-7282.

[104] Furthermore, in one example, the STRO-1⁺ cells are capable of giving rise to clonogenic CFU-F.

[105] In one example, a significant proportion of the STRO-1+ multipotential cells are capable of differentiation into at least two different germ lines. Non-limiting examples of the lineages to which the multipotential cells may be committed include bone precursor cells; hepatocyte progenitors, which are multipotent for bile duct epithelial cells and hepatocytes; neural restricted cells, which can generate glial cell precursors that progress to oligodendrocytes and astrocytes; neuronal precursors that progress to neurons; precursors for cardiac muscle and cardiomyocytes, glucose-responsive insulin secreting pancreatic beta cell lines. Other lineages include, but are not limited to, odontoblasts, dentin-producing cells and chondrocytes, and precursor cells of the following: retinal pigment epithelial cells, fibroblasts, skin cells such as keratinocytes, dendritic cells, hair follicle cells, renal duct epithelial cells, smooth and skeletal muscle cells, testicular progenitors, vascular endothelial cells, tendon, ligament, cartilage, adipocyte, fibroblast, marrow stroma, cardiac muscle, smooth muscle, skeletal muscle, pericyte, vascular, epithelial, glial, neuronal, astrocyte and oligodendrocyte cells.

[106] In an aspect of the present disclosure, the presently described mesenchymal lineage precursor or stem cells are MSCs. The MSCs may be a homogeneous composition or may be a mixed cell population enriched in MSCs. Homogeneous MSCs cell compositions may be obtained by culturing adherent marrow or periosteal cells, and the MSCs may be identified by specific cell surface markers which are identified with unique monoclonal antibodies. A method for obtaining a cell population enriched in MSCs is described, for example, in U.S. Patent No. 5,486,359. Alternative sources for MSCs include, but are not limited to, blood, skin, cord blood, muscle, fat, bone, and perichondrium.

[107] In another example, the mesenchymal lineage precursor or stem cells are CD29+, CD54+, CD73+, CD90+, CD102+, CD105+, CD106+, CD166+, MHC1+ MSCs (*e.g.* remestemcel-L).

[108] As will be appreciated by those of skill in the art, cultured mesenchymal lineage precursor or stem cells are phenotypically different to cells *in-vivo*. For example, in one embodiment they express one or more of the following markers, CD44, NG2, DC146 and CD140b. Cultured mesenchymal lineage precursor or stem cells are also biologically different to cells *in-vivo*, having a higher rate of proliferation compared to the largely non-cycling (quiescent) cells *in-vivo*.

[109] Mesenchymal lineage precursor or stem cells cultured using the methods of the present disclosure may also be cryopreserved.

Culture expanded MLPSC and conditioned media obtained from the same

[110] In example, culture expanded MLPSCs of the disclosure and/or conditioned media obtained from the same are characterised by expression of an angiogenic marker(s). For example, a culture expanded MLPSC population according to the present disclosure and/or conditioned media obtained from the same can be characterised by increased levels of VEGF, angiogenin, and/or SDF-1 α under culture conditions. In another example, the MLPSC population can be characterised based on an assessment of conditioned media obtained from the MLPSC population under culture conditions. In an example, the conditioned media increases the level endothelial network formation, endothelial network length, and/or endothelial branch length in a population of endothelial cells when said cells are treated with conditioned media obtained from culture expanded MLPSCs. In an example, the increase is determined relative to a control population of MLPSCs. In an example, the control population is a population of MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal calf serum.

[111] In an example, the expanded MLPSC population is characterised by a level of VEGF greater than about 3 ng/ml. In an example, the level of VEGF is between about 3 ng/ml to 4 ng/ml. In an example, the level of VEGF is greater than about 3.1 ng/ml. In an example, level of VEGF is greater than about 3.2 ng/ml. In an example, level of VEGF is greater than about 3.3 ng/ml. In an example, the level of VEGF is greater than about 3.4 ng/ml. In an example, the level of VEGF is greater than about 3.5 ng/ml. In an example, the level of VEGF is between about 3.2 and 3.6 ng/mL. In an example, the level of VEGF is about 3.45 ng/mL.

[112] In an example, the MLPSCs express an increased level of angiogenin relative to a control population. In an example, the expanded MLPSC population is characterised by a level of angiogenin greater than about 1000 pg/ml. In an example, the level of angiogenin is greater than about 1100 pg/ml. In an example, the level of angiogenin is between about 1000 pg/ml and 1200 pg/ml. In an example, the level of angiogenin is between about 1100 pg/ml and 1150 pg/ml. In an example, the level of angiogenin is about 1114 pg/ml.

[113] In an example, the expanded MLPSC population is characterised by a level of SDF-1 α greater than about 3000 ng/ml. In an example, the level of SDF-1 α is greater than about 3100 ng/ml. In an example, the level of SDF-1 α is greater than about 3200 ng/ml. In an example, the level of SDF-1 α is greater than about 3300 ng/ml. In an example, the level of SDF-1 α is greater than about 3400 ng/ml. In an example, the level of SDF-1 α is greater than about 3500 ng/ml. In an example, the level of SDF-1 α is

between about 3000 ng/ml and 3500 ng/ml. In an example, the level of SDF-1 α is between about 3000 ng/ml and 3400 ng/ml. In an example, the level of SDF-1 α is between about 3000 ng/ml and 3300 ng/ml. In an example, the level of SDF-1 α is between about 3100 ng/ml and 3400 ng/ml. In an example, the level of SDF-1 α is between about 3100 ng/ml and 3300 ng/ml.

[114] In an example, the culture expanded MLPSC population is characterised by conditioned media which stimulates endothelial network formation greater than about 0.1 mm²/mm². In an example, the endothelial network formation is between about 0.1 mm²/mm² and 0.2 mm²/mm². In another example, the endothelial network formation is about 0.12 mm²/mm².

[115] In an example, the culture expanded MLPSC population is characterised by conditioned media which stimulates endothelial network length greater than about 4 mm²/mm². In an example, the endothelial network length is between about 4 mm²/mm² and about 6 mm²/mm². In an example, the endothelial network length is about 5 mm²/mm². In an example, the culture expanded MLPSC population is characterised by conditioned media which stimulates endothelial branch length greater than about 12 1/mm². In an example, the endothelial branch length is between about 12 1/mm² and about 17 1/mm². In an example, the endothelial branch length is about 15 1/mm².

[116] In an example, the culture expanded MLPSC population is characterised by an increased level of one or more angiogenic markers relative to a population of MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal serum (e.g. fetal calf serum). In an example, the level of angiogenic marker is increased by about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60 %, or about 70% relative to a population of MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal serum (e.g. fetal calf serum). In an example, the level of angiogenic marker is increased by between about 5% and about 60%. In an example, the level of angiogenic marker is increased by between about 5% and about 40%. In an example, the level of angiogenic marker is increased by about 40%. In an example, the level of angiogenic marker is increased by at least about 5%. In an example, the level of angiogenic marker is increased by at least about 10%. In an example, the level of angiogenic marker is increased relative to a population of MLPSCs that have been culture expanded in cell culture medium that does not contain IFN-gamma or TNF-alpha.

[117] In an example, the expanded MLPSC population is characterised by an increased level of one or more angiogenic markers relative to a population of MLPSCs that have been culture expanded in a cell culture medium that does not comprise newborn

serum. In an example, the level of angiogenic marker is increased by about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60 %, or about 70% relative to a population of MLPSCs that have been culture expanded in a cell culture medium that does not comprise newborn serum. In an example, the level of angiogenic marker is increased by between about 5% and about 60%. In an example, the level of angiogenic marker is increased by between about 5% and about 40%. In an example, the level of angiogenic marker is increased by about 40%. In an example, the level of angiogenic marker is increased by at least about 5%. In an example, the level of angiogenic marker is increased by at least about 10%.

[118] In another example, culture expanded MLPSCs of the disclosure are characterised based on therapeutic efficacy. For example, the MLPSCs may be characterised based on therapeutic efficacy in congenital heart disease. In an example, the MLPSCs are characterised by therapeutic efficacy in hypoplastic left heart syndrome.

[119] In another example, culture expanded MLPSCs are characterised by their capacity to inhibit IL-2RA expression by CD3/CD28-activated PBMCs under culture conditions. In an example, the culture expanded MLPSCs inhibit IL2-RA expression by CD3/CD28-activated PBMCs by at least 60% relative to a control. In another example, the culture expanded MLPSCs inhibit IL2-RA expression by CD3/CD28-activated PBMCs by at least 65% relative to a control. In another example, the culture expanded MLPSCs inhibit IL2-RA expression by CD3/CD28-activated PBMCs by at least 70% relative to a control. In another example the culture expanded MLPSCs inhibit IL2-RA expression by CD3/CD28-activated PBMCs by between 60 and 70% relative to a control.

[120] “Culture expanded” MLPSCs are distinguished from freshly isolated cells in that they have been cultured in cell culture medium and passaged (i.e. sub-cultured).

[121] In an example, freshly isolated cells are culture expanded for about 1 or 2 passages to provide an intermediate population. In an example, freshly isolated cells are culture expanded for 2 passages to provide an intermediate population. In another example, freshly isolated cells are culture expanded for about 1 to 3 passages to provide an intermediate population. In an example, freshly isolated cells are STRO-1+.

[122] Accordingly, in an example, relevant cells are isolated and culture expanded for 2 passages to provide an intermediate MLPSC population. In certain examples, the intermediate MLPSC population is then culture expanded to provide a drug product (DP). For example, DP compositions of the present disclosure are produced by culturing cells from an intermediate cryopreserved MLPSC population or, put another way, a cryopreserved intermediate. In an example, the intermediate cell population can be cultured for three more passages (i.e. 5 passages total) to provide a DP.

[123] In an example, MLPSCs are culture expanded for about 4 – 10 passages. In an example, MLPSCs are culture expanded for at least 5, at least 6, at least 7, at least 8, at least 9, at least 10 passages. For example, MLPSCs can be culture expanded for at least 5 passages. In an example, MLPSCs can be culture expanded for at least 5 – 10 passages. In an example, MLPSCs can be culture expanded for at least 5 – 8 passages. In an example, MLPSCs can be culture expanded for at least 5 – 7 passages. In an example, MLPSCs can be culture expanded for more than 7 passages. In these examples, MLPSCs may be culture expanded before being cryopreserved to provide an intermediate cryopreserved MLPSC population and then subject to further culture expansion.

[124] In an example, compositions of the disclosure comprise MLPSCs that are culture expanded from a cryopreserved intermediate. In an example, the cells culture expanded from a cryopreserved intermediate are culture expanded for at least 3, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10 passages. For example, MLPSCs can be culture expanded for at least 3 passages. In an example, MLPSCs can be culture expanded for at least 3 – 10 passages. In an example, MLPSCs can be culture expanded for at least 3 – 8 passages. In an example, MLPSCs can be culture expanded for at least 3 – 7 passages. In an example, MLPSCs culture expanded from a cryopreserved intermediate are culture expanded in media disclosed herein (e.g. media containing newborn calf serum).

[125] In an example, MLPSCs can be obtained from a single donor, or multiple donors where the donor samples or MLPSCs are subsequently pooled and then culture expanded as required. In an example, the culture expansion process comprises:

- i. expanding by passage expansion the number of viable cells to provide a preparation of at least about 1 billion of the viable cells, wherein the passage expansion comprises establishing a primary culture of isolated MLPSCs and then serially establishing a first non-primary (P1) culture of isolated MLPSCs from the previous culture;
- ii. expanding by passage expansion the P1 culture of isolated MLPSCs to a second non-primary (P2) culture of MLPSCs; and,
- iii. preparing and cryopreserving an in-process intermediate MLPSC preparation obtained from the P2 culture of MLPSCs; and, optionally
- iv. thawing the cryopreserved in-process intermediate MLPSC preparation and expanding by passage expansion the in-process intermediate MLPSC preparation.

[126] In an example, the methods of the disclosure comprise selecting an intermediate population (e.g. a cryopreserved intermediate) for further culture expansion based on certain criteria such as the level of one more angiogenic markers. Selection processes

are not particularly limited so long as they are able to select cell populations characterized by the relevant criteria such as level of angiogenic marker. In an example, a series of intermediate MLPSC populations are assessed for levels of angiogenic markers and those populations which express over a threshold level of the angiogenic marker as described herein are selected for further expansion.

[127] It should be appreciated that the selection process does not require immediate culture expansion. Rather “selected” populations can be cryopreserved and culture expanded at a later stage. In an example, a fraction of the intermediate cell population is culture expanded with the remainder of the population being cryopreserved for culture expansion at a later stage.

[128] In an example, selected cell populations are immediately culture expanded. In another example, selected cell populations are cryopreserved to allow culture expansion at a later stage.

[129] In an example, a selected cell population is culture expanded to provide a pharmaceutical composition. In an example, the pharmaceutical composition is characterized by certain criteria such as level of angiogenic markers.

[130] In the context of the present disclosure, the level of angiogenic marker/s can be assessed between steps iii and iv of the culture expansion process described above. For example, the level of angiogenic marker/s may be determined under culture conditions and/or from conditioned media after step iii. In an example, step iv is only performed if a desired level of angiogenic marker/s is/are observed under culture conditions and/or from conditioned media. In this example, the cell population is selected for culture expansion on the basis of the level of angiogenic marker/s under culture conditions and/or from conditioned media.

[131] In an example, the culture expanded MLPSC population is expanded from an intermediate MLPSC population with an increased level of one or more angiogenic markers relative to a population of MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal calf serum.

[132] In an example, a level of an angiogenic marker(s) disclosed herein is considered increased when it is increased by about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60 %, or about 70% relative to a population of MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal calf serum. In an example, the level of angiogenic marker is increased by between about 5% and about 60%. In an example, the level of angiogenic marker is increased by between about 5% and about 40%. In an example, the level of angiogenic marker is increased by about

40%. In an example, the level of angiogenic marker is increased by at least about 5%. In an example, the level of angiogenic marker is increased by at least about 10%.

[133] In an example, the culture expanded MLPSC population is expanded from an intermediate MLPSC population with an increased level of one or more angiogenic markers relative to a population of MLPSCs that have been culture expanded in a cell culture medium that does not comprise newborn serum. In an example, the level of angiogenic marker is considered increased when it is increased by about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60 %, or about 70% relative to a population of MLPSCs that have been culture expanded in a cell culture medium that does not comprise newborn serum. In an example, the level of angiogenic marker is increased by between about 5% and about 60%. In an example, the level of angiogenic marker is increased by between about 5% and about 40%. In an example, the level of angiogenic marker is increased by about 40%. In an example, the level of angiogenic marker is increased by at least about 5%. In an example, the level of angiogenic marker is increased by at least about 10%.

[134] In an example, the culture expanded MLPSC preparation has an antigen profile and an activity profile comprising:

- i. less than about 0.75% CD45+ cells;
- ii. at least about 95% CD105+ cells;
- iii. at least about 95% CD166+ cells.

Conditioned media

[135] In an example, conditioned media or extracellular vesicles obtained therefrom are characterised by expression of an angiogenic marker(s). For example, conditioned media or extracellular vesicles obtained therefrom can be characterised by increased levels of VEGF, angiogenin, and/or SDF-1 α under culture conditions. In another example, conditioned media or extracellular vesicles obtained therefrom can be characterised based on one or more functional criteria. In an example, the conditioned media or extracellular vesicles obtained therefrom increases the level endothelial network formation, endothelial network length, and/or endothelial branch length in a population of endothelial cells when said cells are treated with conditioned media or extracellular vesicles obtained therefrom that have been obtained from culture expanded MLPSCs. In an example, the increase is determined relative to conditioned media or extracellular vesicles obtained therefrom which have been obtained from a control population of MLPSCs. In an example, the control population is a population of

MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal calf serum.

[136] In an example, the conditioned media is characterised by a level of VEGF greater than about 3 ng/ml. In an example, the level of VEGF is between about 3 ng/ml to 4 ng/ml. In an example, the level of VEGF is greater than about 3.1 ng/ml. In an example, level of VEGF is greater than about 3.2 ng/ml. In an example, level of VEGF is greater than about 3.3 ng/ml. In an example, the level of VEGF is greater than about 3.4 ng/ml. In an example, the level of VEGF is greater than about 3.5 ng/ml. In an example, the level of VEGF is between about 3.2 and 3.6 ng/mL. In an example, the level of VEGF is about 3.45 ng/mL.

[137] In an example, the conditioned media or extracellular vesicles obtained therefrom contain an increased level of angiogenin relative to a control population. In an example, the conditioned media is characterised by a level of angiogenin greater than about 1000 pg/ml. In an example, the level of angiogenin is greater than about 1100 pg/ml. In an example, the level of angiogenin is between about 1000 pg/ml and 1200 pg/ml. In an example, the level of angiogenin is between about 1100 pg/ml and 1150 pg/ml. In an example, the level of angiogenin is about 1114 pg/ml.

[138] In an example, the conditioned media is characterised by a level of SDF-1 α greater than about 3000 ng/ml. In an example, the level of SDF-1 α is greater than about 3100 ng/ml. In an example, the level of SDF-1 α is greater than about 3200 ng/ml. In an example, the level of SDF-1 α is greater than about 3300 ng/ml. In an example, the level of SDF-1 α is greater than about 3400 ng/ml. In an example, the level of SDF-1 α is greater than about 3500 ng/ml. In an example, the level of SDF-1 α is between about 3000 ng/ml and 3500 ng/ml. In an example, the level of SDF-1 α is between about 3000 ng/ml and 3400 ng/ml. In an example, the level of SDF-1 α is between about 3000 ng/ml and 3300 ng/ml. In an example, the level of SDF-1 α is between about 3100 ng/ml and 3400 ng/ml. In an example, the level of SDF-1 α is between about 3100 ng/ml and 3300 ng/ml.

[139] In an example, the conditioned media stimulates endothelial network formation greater than about 0.1 mm²/mm². In an example, the endothelial network formation is between about 0.1 mm²/mm² and 0.2 mm²/mm². In another example, the endothelial network formation is about 0.12 mm²/mm².

[140] In an example, the conditioned media stimulates endothelial network length greater than about 4 mm²/mm². In an example, the endothelial network length is between about 4 mm²/mm² and about 6 mm²/mm². In an example, the endothelial network length is about 5 mm²/mm². In an example, the conditioned media stimulates endothelial branch length greater than about 12 1/mm². In an example, the endothelial branch length is

between about 12 1/mm^2 and about 17 1/mm^2 . In an example, the endothelial branch length is about 15 1/mm^2 .

[141] In an example, the conditioned media or extracellular vesicles obtained therefrom is characterised by an increased level of one or more angiogenic markers relative to conditioned media or extracellular vesicles obtained therefrom that have been obtained from a population of MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal calf serum. In an example, the level of angiogenic marker is increased by about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60 %, or about 70%. In an example, the level of angiogenic marker is increased by between about 5% and about 60%. In an example, the level of angiogenic marker is increased by between about 5% and about 40%. In an example, the level of angiogenic marker is increased by about 40%. In an example, the level of angiogenic marker is increased by at least about 5%. In an example, the level of angiogenic marker is increased by at least about 10%. In an example, the level of angiogenic marker is increased relative to conditioned media or extracellular vesicles obtained therefrom that have been obtained from a population of MLPSCs that have been culture expanded in cell culture medium that does not contain IFN-gamma or TNF-alpha.

[142] In an example, the conditioned media or extracellular vesicles obtained therefrom is characterised by an increased level of one or more angiogenic markers relative to conditioned media or extracellular vesicles obtained therefrom that have been obtained from a population of MLPSCs that have been culture expanded in a cell culture medium that does not comprise newborn serum. In an example, the level of angiogenic marker is increased by about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60 %, or about 70%. In an example, the level of angiogenic marker is increased by between about 5% and about 60%. In an example, the level of angiogenic marker is increased by between about 5% and about 40%. In an example, the level of angiogenic marker is increased by about 40%. In an example, the level of angiogenic marker is increased by at least about 5%. In an example, the level of angiogenic marker is increased by at least about 10%.

[143] In an example, freshly isolated cells are culture expanded for about 1 or 2 passages to provide an intermediate population. In an example, freshly isolated cells are culture expanded for 2 passages to provide an intermediate population. In another example, freshly isolated cells are culture expanded for about 1 to 3 passages to provide an intermediate population. In an example, freshly isolated cells are STRO-1+. In an example, conditioned media or extracellular vesicles obtained therefrom are produced by culture expanding cells to provide a cryopreserved intermediate.

[144] Accordingly, in an example, relevant cells are isolated and culture expanded for 2 passages to provide an intermediate MLPSC population. In certain examples, the intermediate MLPSC population is then culture expanded to provide a drug product (DP). In an example, conditioned media or extracellular vesicles obtained therefrom are obtained from DP MLPSCs.

[145] In an example, MLPSCs are culture expanded for about 4 – 10 passages to provide conditioned media or extracellular vesicles obtained therefrom. In an example, MLPSCs are culture expanded for at least 5, at least 6, at least 7, at least 8, at least 9, at least 10 passages to provide conditioned media or extracellular vesicles obtained therefrom. For example, MLPSCs can be culture expanded for at least 5 passages to provide conditioned media or extracellular vesicles obtained therefrom. In an example, MLPSCs can be culture expanded for at least 5 – 10 passages to provide conditioned media or extracellular vesicles obtained therefrom. In an example, MLPSCs can be culture expanded for at least 5 – 8 passages to provide conditioned media or extracellular vesicles obtained therefrom. In an example, MLPSCs can be culture expanded for at least 5 – 7 passages to provide conditioned media or extracellular vesicles obtained therefrom. In an example, MLPSCs can be culture expanded for more than 7 passages to provide conditioned media or extracellular vesicles obtained therefrom. In these examples, MLPSCs may be culture expanded before being cryopreserved to provide an intermediate cryopreserved MLPSC population and then subject to further culture expansion to provide conditioned media or extracellular vesicles obtained therefrom.

[146] In an example, conditioned media or extracellular vesicles obtained therefrom are obtained from MLPSCs that have been culture expanded from a cryopreserved intermediate. In an example, the cells culture expanded from a cryopreserved intermediate are culture expanded for at least 3, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10 passages. For example, MLPSCs can be culture expanded for at least 3 passages. In an example, MLPSCs can be culture expanded for at least 3 – 10 passages. In an example, MLPSCs can be culture expanded for at least 3 – 8 passages. In an example, MLPSCs can be culture expanded for at least 3 – 7 passages. In an example, MLPSCs culture expanded from a cryopreserved intermediate are culture expanded in media disclosed herein (e.g. media containing newborn calf serum).

[147] In an example, MLPSCs can be obtained from a single donor, or multiple donors where the donor samples or MLPSCs are subsequently pooled and then culture expanded as required. In an example, the culture expansion process comprises:

- i. expanding by passage expansion the number of viable cells to provide a preparation of at least about 1 billion of the viable cells, wherein the passage expansion

comprises establishing a primary culture of isolated MLPSCs and then serially establishing a first non-primary (P1) culture of isolated MLPSCs from the previous culture;

ii. expanding by passage expansion the P1 culture of isolated MLPSCs to a second non-primary (P2) culture of MLPSCs; and,

iii. preparing and cryopreserving an in-process intermediate MLPSC preparation obtained from the P2 culture of MLPSCs; and, optionally

iv. thawing the cryopreserved in-process intermediate MLPSC preparation and expanding by passage expansion the in-process intermediate MLPSC preparation.

[148] In an example, the methods of the disclosure comprise selecting an intermediate population (e.g. a cryopreserved intermediate) for further culture expansion based on certain criteria such as the level of one more angiogenic markers. Selection processes are not particularly limited so long as they are able to select cell populations characterized by the relevant criteria such as level of angiogenic marker. In an example, a series of intermediate MLPSC populations are assessed for levels of angiogenic markers and those populations which express over a threshold level of the angiogenic marker as described herein are selected for further expansion.

[149] It should be appreciated that the selection process does not require immediate culture expansion. Rather “selected” populations can be cryopreserved and culture expanded at a later stage. In an example, a fraction of the intermediate cell population is culture expanded with the remainder of the population being cryopreserved for culture expansion at a later stage.

[150] In an example, selected cell populations are immediately culture expanded. In another example, selected cell populations are cryopreserved to allow culture expansion at a later stage.

[151] In an example, conditioned media or extracellular vesicles obtained therefrom are obtained from a culture expanded MLPSC population that has an antigen profile and an activity profile comprising:

- i. less than about 0.75% CD45+ cells;
- ii. at least about 95% CD105+ cells;
- iii. at least about 95% CD166+ cells.

General MLPSC expansion methods

[152] The process of MLPSC isolation and *ex vivo* expansion can be performed using any equipment and cell handling methods known in the art. Various culture expansion embodiments of the present disclosure employ steps that require manipulation of cells,

for example, steps of seeding, feeding, dissociating an adherent culture, or washing. Any step of manipulating cells has the potential to insult the cells. Although MLPSCs can generally withstand a certain amount of insult during preparation, cells are preferably manipulated by handling procedures and/or equipment that adequately performs the given step(s) while minimizing insult to the cells.

[153] In an example, MLPSCs are washed in an apparatus that includes a cell source bag, a wash solution bag, a recirculation wash bag, a spinning membrane filter having inlet and outlet ports, a filtrate bag, a mixing zone, an end product bag for the washed cells, and appropriate tubing, for example, as described in US 6,251,295, which is hereby incorporated by reference.

[154] In an example, a MLPSC composition cultured according to the present disclosure is 95% homogeneous with respect to being CD105 positive and CD166 positive and being CD45 negative. In an example, this homogeneity persists through *ex vivo* expansion; i.e. through multiple population doublings.

[155] In an example, MLPSCs of the disclosure are culture expanded in 2D culture. For example, MLPSCs of the disclosure can be culture expanded in a cell factory. In certain examples, 3D culture of intermediates disclosed herein may follow using, for example, a bioreactor. In an example, MLPSCs of the disclosure are initially culture expanded in 2D culture prior to being further expanded in 3D culture. In an example, intermediate cell populations of the disclosure have not been culture expanded in 3D culture. In an example, the level of one or more angiogenic markers is assessed before subsequent culture expansion in a cell factory or 3D culture.

[156] In an example, MLPSCs of the disclosure are culture expanded from an intermediate population. In an example, MLPSCs of the disclosure are culture expanded from the intermediate in 2D culture before seeding in 3D culture.

[157] In the context of both intermediate populations and therapeutic compositions expanded from the same, in an example, MLPSCs of the disclosure are culture expanded in 2D culture for at least 3 days before seeding in a further culture system such as cell factory or 3D culture in a bioreactor. In an example, MLPSCs of the disclosure are culture expanded in 2D culture for at least 4 days before seeding in a further culture system. In an example, MLPSCs of the disclosure are culture expanded in 2D culture for between 3 and 5 days before seeding in a further culture system. In these examples, 2D culture can be performed in a cell factory. Various cell factory products are available commercially (e.g. Thermofisher, Sigma, Corning). In an example, the cell factory has at least 5 layers. In an example, the cell factory has at least 10 layers. In an example,

the cell factory has at least 20 layers. 3D culture may be performed in various bioreactor types such as stirred tank, wave bag, and vertical wheel.

[158] In an example, CO₂ is provided during culture expansion of MLPSCs. In an example, MLPSCs are culture expanded in less than 9% CO₂. In an example, MLPSCs are culture expanded in less than 8% CO₂. In an example, MLPSCs are culture expanded in 5% CO₂. For example, MLPSCs can be culture expanded in 5% +/- 2% CO₂. In an example, the MLPSCs are culture expanded with passive priming of CO₂. For example, cell factories can be passively primed with 5% CO₂.

Priming cell factories maintains the CO₂ tension between the cell factory and incubator and stabilizes the pH level of the growth medium. Active priming involves actively passing CO₂ gas through a bacterial vent air filter into each culture vessel (e.g. cell factory) for a defined period of time (e.g. around 10 minutes). However, active priming has the potential to introduce contamination into culture as it requires an open port to provide gas. Passive priming involves placing a closed culture system into an incubator at appropriate CO₂ concentration prior to cell seeding (e.g. around 12 to 72 hours). In an example, cells of the disclosure are STRO-3+ before they are culture expanded to provide an intermediate cell population.

Cell Culture Methods

[159] In certain examples, compositions of the disclosure can be prepared via culture expansion in media containing one or more pro-inflammatory cytokines and/or a non-fetal serum disclosed herein, such as newborn serum.

[160] For example, MLPSC culture media can be supplemented with pro-inflammatory cytokine(s). In an example, the culture media comprises IFN-gamma and/or TNF-alpha. In an example, the media comprises IFN-gamma. For example, the level of IFN-gamma can be less than 1 ng/ml. In an example the level of IFN-gamma is less than 500 pg/ml or less than 100 pg/ml. In an example, the media comprises TNF-alpha. For example, the level of TNF-alpha can be less than 1 ng/ml. In an example, the level of TNF-alpha is less than 750 pg/ml or less than 400 pg/ml. In an example, the media comprises IFN-gamma and TNF-alpha and the level of both is less than 1 ng/ml.

[161] In an example, the media comprises one or more pro-inflammatory cytokines which are capable of binding a receptor on the surface of MLPSCs.

[162] In an example, the media comprises one or more pro-inflammatory cytokines selected from the group consisting of IL-6; IL-8; IL-17A; MCP-1; MIP-1-alpha; MIP-1-beta; IP-10. For example, the media can comprise IL-8.

[163] In an example, the media comprises IFN-gamma and/or TNF-alpha, and, one or more pro-inflammatory cytokines selected from the group consisting of IL-6; IL-8; IL-17A; MCP-1; MIP-1-alpha; MIP-1-beta; IP-10. In an example, the level of IFN-gamma and/or TNF-alpha is less than 1 ng/ml.

[164] In an example, the media is characterised by one or more or all of the following:

- i. a level of IFN-gamma greater than 1 pg/ml;
- ii. a level of TNF-alpha greater than 2 pg/ml;
- iii. a level of IL-6 greater than 3 pg/ml;
- iv. a level of IL-8 greater than 500 pg/ml;
- v. a level of IL-17A greater than 0.2 pg/ml;
- vi. a level of MCP-1 greater than 3 pg/ml;
- vii. a level of MIP-1-alpha greater than 0.5 pg/ml;
- viii. a level of MIP-1-beta greater than 3 pg/ml;
- ix. a level of IP-10 greater than 500 pg/ml.

[165] In another example, the media comprises serum which is characterised by one or more or all of the following:

- i. a level of IFN-gamma greater than 10 pg/ml;
- ii. a level of TNF-alpha greater than 20 pg/ml;
- iii. a level of IL-6 greater than 30 pg/ml;
- iv. a level of IL-8 greater than 5,000 pg/ml;
- v. a level of IL-17A greater than 2 pg/ml;
- vi. a level of MCP-1 greater than 30 pg/ml;
- vii. a level of MIP-1-alpha greater than 50 pg/ml;
- viii. a level of MIP-1-beta greater than 30 pg/ml;
- ix. a level of IP-10 greater than 5,000 pg/ml.

[166] In an example, the media comprises IL-10. In another example, the media comprises IL-36RA. In another example, the media comprises IL-10 and IL-36RA. In an example, the level of IL-10 is greater than 0.3 pg/ml. For example, the level of IL-10 may be greater than 30 pg/ml. In an example, the level of IL-10 is greater than 400 pg/ml. In an example, the level of IL-36RA is greater than 50 pg/ml.

[167] In an example, the media is serum free.

[168] In an example, the media is serum free and supplemented with PDGF and FGF2. In an example, the medium is serum free and is supplemented with PDGF, FGF2 and

EGF. In an example, the PDGF is PDGF-BB. In an example, the serum free media is supplemented with 10 ng/ml PDGF-BB, 5 ng/ml EGF and, 1 ng/ml FGF2.

[169] In an example, the above referenced cytokines can be provided at a concentration < 1 ng/ml each. For example, the media may be characterised by one or more or all of the following, each provided at < 1ng/ml: IFN-gamma, TNF-alpha, IL-6, IL-17A, MCP-1, MIP-1-alpha, MIP-1-beta, IP-10.

[170] In another example, compositions of the disclosure can be prepared via culture expansion in a culture medium that is supplemented with a serum comprising one or more pro-inflammatory cytokines as described herein. In some preferred embodiments, the culture medium is supplemented with a non-fetal serum, such as newborn serum. In some preferred embodiments, the culture medium is supplemented with both fetal serum and newborn serum in equal concentrations for a total serum concentration in the culture medium of about 10% (v/v). In some preferred embodiments MLPSCs are pre-licensed in cell culture medium containing 5% (v/v) newborn serum and 5% (v/v) fetal serum.

[171] In some embodiments the methods of preparing MLPSCs disclosed herein include the additional step of determining or having determined the level of one or more pro-inflammatory cytokines in a serum to be included in the culture medium to be used for pre-licensing of MLPSCs. Methods for determining cytokine levels are well known in the art, *e.g.*, ELISA.

[172] In some embodiments the methods of preparing MLPSCs disclosed herein also include determining or having determined the ability of a culture medium (*e.g.* a newborn serum supplemented culture medium) to stimulate MLPSCs to promote angiogenesis in an *in vitro* assay, *e.g.*, tube formation by human umbilical vein endothelial cells (HUVEC) and analysis of network length, network area and branch point formation. In some embodiments such an assay includes collecting MLPSC-conditioned media following its culture in a newborn serum-supplemented medium as disclosed herein and quantifying the effect of such conditioned media in the above-described angiogenesis assay or a similar assay.

[173] In some embodiments the methods of preparing MLPSCs disclosed herein also include determining or having determined in the above-mentioned conditioned medium the level of one or more of Angiogenin, Angiopoietin (Ang1/ANGPT1), SDF-1 α , and VEGF.

[174] In some embodiments, where a first lot or batch of newborn serum was used in conditioned medium that promotes greater angiogenesis or release of angiogenic factors than that of conditioned medium in which second lot/batch of newborn serum was used, it is concluded that the use of the first lot of newborn serum for pre-licensing and culture

expansion of MLPSCs will result in the generation of MLPSCs that have relatively greater therapeutic potency particularly for treatment of conditions where an angiogenic or anti-inflammatory therapeutic mode of action is useful.

[175] The methods and cell culture media used to prepare MLPSCs of the disclosure promote stem cell proliferation while maintaining MLPSCs in an undifferentiated state. MLPSCs are considered to be undifferentiated when they have not committed to a specific differentiation lineage. As discussed above, MLPSCs display morphological characteristics that distinguish them from differentiated cells. Furthermore, undifferentiated MLPSCs express genes that may be used as markers to detect differentiation status. The polypeptide products may also be used as markers to detect differentiation status. Accordingly, one of skill in the art could readily determine whether the methods of the present disclosure maintain MLPSCs in an undifferentiated state using routine morphological, genetic and/or proteomic analysis. Methods of monitoring/confirming cell proliferation are also known in the art and, in certain examples, may be as rudimentary as periodic visual inspection of cell cultures to confirm increase in cell number. Other methods may involve the use of cell viability dyes and/or live cell imaging and counting using commercially available products.

[176] MLPSCs disclosed herein can be culture expanded in various suitable cell culture mediums comprising newborn serum. The term “medium” or “media” as used in the context of the present disclosure, includes the components of the environment surrounding the cells. The media contributes to and/or provides the conditions suitable to allow cells to grow. Media may be solid, liquid, gaseous or a mixture of phases and materials. Media can include liquid growth media as well as liquid media that do not sustain cell growth. Exemplary gaseous media include the gaseous phase that cells growing on a petri dish or other solid or semisolid support are exposed to.

[177] The cell culture media used for culture expansion contains all essential amino acids and may also contain non-essential amino acids. In general, amino acids are classified into essential amino acids (Thr, Met, Val, Leu, Ile, Phe, Trp, Lys, His) and non-essential amino acids (Gly, Ala, Ser, Cys, Gln, Asn, Asp, Tyr, Arg, Pro).

[178] Those of skill in the art will appreciate that for optimal results, the basal medium must be appropriate for the cell line of interest. For example, it may be necessary to increase the level of glucose (or other energy source) in the basal medium, or to add glucose (or other energy source) during the course of culture, if this energy source is found to be depleted and to thus limit growth. In an example, dissolved oxygen (DO) levels can also be controlled.

[179] In the above examples, basal medium such as Alpha MEM or StemSpan™ can be supplemented with the referenced quantity of serum and, in certain examples, other additives. Further examples of suitable culture mediums for culturing stem cells can be found, for example, in WO2016139340.

Serum

[180] In an example, MLPSCs of the disclosure are culture expanded in media containing fetal serum (e.g. 10% fetal serum). In other example, MLPSCs are culture expanded in media supplemented with non-fetal serum.

[181] “Non-fetal serum” refers to serum that has been obtained postpartum. For example, the culture media can be supplemented with mammalian non-fetal serum (e.g. bovine). In an example, the culture media can be supplemented with an animal non-fetal serum. In another example, the culture media can be supplemented with human non-fetal serum.

[182] In an example, the cell culture media is supplemented with at least about 1% v/v, at least about 2% v/v, at least about 3% v/v, at least about 4% v/v, at least about 5% v/v, at least about 6% v/v, at least about 7% v/v, at least about 8% v/v, at least about 9%, at least about 10%, at least about 11%, at least about 12%, at least about 13%, at least about 14%, at least about 15%, at least about 16%, at least about 17%, at least about 18%, at least about 19%, at least about 20%, at least about 21%, at least about 22%, at least about 23%, at least about 24%, at least about 25% v/v non-fetal serum. In an example, the cell culture media is supplemented with between about 1% v/v and about 15% v/v non-fetal serum. In an example, the cell culture media is supplemented with between about 1% v/v and about 10% v/v non-fetal serum. In an example, the cell culture media is supplemented with between about 5% v/v and about 10% v/v non-fetal serum. In an example, the cell culture media is supplemented with between about 5% v/v non-fetal serum.

[183] In an example, the non-fetal serum comprises at least one pro-inflammatory cytokine. Methods to detect the presence of cytokines in cell culture medium and/or serum are known in the art and include, for example, enzyme-linked immunosorbent assay (ELISA). In another example, the presence of cytokines in serum are detected by measuring cytokine mRNA, for example by polymerase-chain reaction (PCR) techniques such as reverse-transcription PCR.

[184] In an example, the non-fetal serum is a newborn serum such as newborn calf serum. “Newborn serum” refers to serum that has been obtained postpartum. For example, the culture media can be supplemented with mammalian newborn serum (e.g.

bovine). In an example, the culture media can be supplemented with animal newborn serum.

[185] In an example, the newborn serum is obtained within 4 weeks after birth of the animal. In an example, the newborn serum is obtained within 21 days after birth of the animal. For example, the newborn serum is obtained ≤ 21 days after birth of the animal. In an example, the newborn serum is obtained between the day of birth and 21 days after birth of the animal. In an example, the newborn serum is obtained between the day of birth and 14 days after birth of the animal. In an example, the newborn serum is obtained between the day of birth and 10 days after birth of the animal. In an example, the newborn serum is obtained between the day of birth and 7 days after birth of the animal. In an example, the newborn serum is obtained between 6 hours after birth and 72 hours after birth. In an example, the newborn serum is obtained between 6 hours after birth and 48 hours after birth. In an example, the newborn serum is obtained between 6 hours after birth and 24 hours after birth. In an example, the newborn serum is obtained between 12 hours after birth and 24 hours after birth.

[186] In an example, the cell culture media is supplemented with at least about 1% v/v, at least about 2% v/v, at least about 3% v/v, at least about 4% v/v, at least about 5% v/v, at least about 6% v/v, at least about 7% v/v, at least about 8% v/v, at least about 9%, at least about 10%, at least about 11%, at least about 12%, at least about 13%, at least about 14%, at least about 15%, at least about 16%, at least about 17%, at least about 18%, at least about 19%, at least about 20%, at least about 21%, at least about 22%, at least about 23%, at least about 24%, at least about 25% v/v newborn serum. In an example, the cell culture media is supplemented with between about 1% v/v and about 15% v/v newborn serum. In an example, the cell culture media is supplemented with between about 1% v/v and about 10% v/v newborn serum. In an example, the cell culture media is supplemented with between about 5% v/v and about 10% v/v newborn serum. In an example, the cell culture media is supplemented with about 5% v/v newborn serum.

[187] In an example, the newborn serum comprises at least one inflammatory cytokine. As used herein, the term “inflammatory cytokine” refers to a signalling molecule that promotes inflammation. In example, the one or more cytokine is selected from the group comprising IL-1 β , IL-6, TNF- α , IFN- γ and/or IL-1ra.

[188] In an example, the newborn serum comprises IFN-gamma. In another example, the newborn serum comprises TNF-alpha. In another example, the newborn serum comprises IFN-gamma and TNF-alpha. In another example, the newborn serum comprises one or more pro-inflammatory cytokines selected from the group consisting of IL-6; IL-8; IL-17A; MCP-1; MIP-1-alpha; MIP-1-beta; IP-10. For example, the

newborn serum can comprise IL-8. In an example, the newborn serum comprises IFN-gamma and/or TNF-alpha and, one or more pro-inflammatory cytokines selected from the group consisting of IL-6; IL-8; IL-17A; MCP-1; MIP-1-alpha; MIP-1-beta; IP-10. In another example, the newborn serum comprises IFN-gamma and TNF-alpha and, one or more pro-inflammatory cytokines selected from the group consisting of IL-6; IL-8; IL-17A; MCP-1; MIP-1-alpha; MIP-1-beta; IP-10. In an example, the level of IFN-gamma is less than 1 ng/ml. In an example, the level of TNF-alpha is less than 1 ng/ml. In an example, the level of both IFN-gamma and TNF-alpha are less than 1 ng/ml. For example, the level of IFN-gamma may be less than 500 pg/ml or less than 100 pg/ml. In an example, the level of TNF-alpha is less than 750 pg/ml or less than 400 pg/ml.

[189] Methods to detect the presence of cytokines in serum are known in the art and include, for example, enzyme-linked immunosorbent assay (ELISA). In another example, the presence of cytokines in serum are detected by measuring cytokine mRNA, for example by polymerase-chain reaction (PCR) techniques such as reverse-transcription PCR.

[190] In an example, the newborn serum can be newborn calf serum (NBCS). In an example, the NBCS is obtained from newborn calves who have been fed colostrum. In an example, NBCS comprises elevated levels of at least one inflammatory cytokine relative to NBCS obtained from a calf that has not been fed colostrum. In an example, NBCS comprises elevated levels of at least one inflammatory cytokine relative to fetal serum such as FCS.

[191] In an example, the NBCS is obtained within 4 weeks after birth of the calf. In an example, the NBCS is obtained within 21 days after birth of the calf. For example, the NBCS is obtained ≤ 21 days after birth of the calf. In an example, the NBCS is obtained between the day of birth and 21 days after birth of the calf. In an example, the NBCS is obtained between the day of birth and 14 days after birth of the calf. In an example, the NBCS is obtained between the day of birth and 10 days after birth of the calf. In an example, the NBCS is obtained between the day of birth and 7 days after birth of the calf. In an example, the NBCS is obtained between 6 hours after birth and 72 hours after birth. In an example, the NBCS is obtained between 6 hours after birth and 48 hours after birth. In an example, the NBCS is obtained between 6 hours after birth and 24 hours after birth. In an example, the NBCS is obtained between 12 hours after birth and 24 hours after birth.

[192] In an example, the cell culture media is supplemented with at least about 1% v/v, at least about 2% v/v, at least about 3% v/v, at least about 4% v/v, at least about 5% v/v, at least about 6% v/v, at least about 7% v/v, at least about 8% v/v, at least about 9%,

at least about 10%, at least about 11%, at least about 12%, at least about 13%, at least about 14%, at least about 15%, at least about 16%, at least about 17%, at least about 18%, at least about 19%, at least about 20%, at least about 21%, at least about 22%, at least about 23%, at least about 24%, at least about 25% v/v NBCS. In an example, the cell culture media is supplemented with between about 1% v/v and about 15% v/v NBCS. In an example, the cell culture media is supplemented with between about 5% v/v and about 10% v/v NBCS. In an example, the cell culture media is supplemented with at least about 5% v/v NBCS.

[193] In an example, the culture medium is also supplemented with fetal serum. In an example, the fetal serum is fetal calf serum (FCS). It is envisaged that the term fetal calf serum (FCS) and fetal bovine serum (FBS) can in the context of the present disclosure be used interchangeably. In an example, cell culture medium is supplemented with less than 10% v/v FCS. In an example, cell culture medium is supplemented with about 5% v/v FCS.

[194] In an example, the culture media is supplemented with a mixture of FCS and NBCS. In an example the cell culture medium is supplemented with about 5% v/v FCS and about 5% v/v NBCS (i.e. a 1:1 ratio of FCS to NBCS). In an example, the culture media can be supplemented with a mixture of FCS and NBCS so that the FCS:NBCS ratio is at least about 0.4:1, at least about 0.5:1, at least about 0.6:1, at least about 0.7:1, at least about 0.8:1, at least about 0.9:1, at least about 1:1, at least about 1.5:1, at least about 2:1. In an example, the FCS:NBCS ratio is between about 0.5:1 and about 2:1. In an example, the FCS:NBCS ratio is between about 0.8:1 and about 1.5:1. In an example, the FCS:NBCS ratio is between about 0.8:1 and about 1.2:1. In an example, the FCS:NBCS ratio is about 1:1.

[195] In an example, the mixture of FCS and NBCS can comprise at least about 1% v/v, at least about 2% v/v, at least about 3% v/v, at least about 4% v/v, at least about 5% v/v, at least about 6% v/v, at least about 7% v/v, at least about 8% v/v, at least about 9%, at least about 10%, at least about 11%, at least about 12%, at least about 13%, at least about 14%, at least about 15%, at least about 16%, at least about 17%, at least about 18%, at least about 19%, at least about 20%, at least about 21%, at least about 22%, at least about 23%, at least about 24%, at least about 25% v/v of the cell culture media. In an example, the mixture of FCS and NBCS can comprise between about 1% v/v and about 15% v/v of the cell culture media. In an example, the mixture of FCS and NBCS can comprise between about 2% v/v and about 12% v/v of the cell culture media. In an example, the mixture of FCS and NBCS can comprise between about 5% v/v and about 12% v/v of the cell culture media. In an example, the mixture of FCS and NBCS can

comprise between about 8% v/v and about 12% v/v of the cell culture media. In an example, the mixture of FCS and NBCS can comprise about 10% v/v of the cell culture media. However, in this example, the cell culture media is supplemented with at least about 1% v/v, at least about 2% v/v, at least about 3% v/v, at least about 4% v/v, at least about 5% v/v, at least about 6% v/v, at least about 7% v/v, at least about 8% v/v, at least about 9% v/v, but less than 10% v/v FCS. In an example, the cell culture media is supplemented with between about 1% v/v and about 9% v/v FCS. In an example, the cell culture media is supplemented with between about 3% v/v and about 8% v/v FCS. In an example, the cell culture media is supplemented with between about 3% v/v and about 6% v/v FCS. In an example, the cell culture media is supplemented with about 5% v/v FCS.

Ascorbic acid

[196] In an example, the cell culture media is supplemented with a short acting ascorbic acid derivative. The term “short acting” encompasses ascorbic acid derivatives that are oxidised by approximately 80 - 90 % following 24 hours of cell culture under culture conditions of neutral pH and 37 °C. In one example, the short acting L-ascorbic acid derivative is a L-ascorbic acid salt, for example L-ascorbic acid sodium salt. In an example, the cell culture media may contain at least about 0.005 g/L of a short acting ascorbic acid derivative. In another example, the cell culture media may contain at least about 0.01 g/L of a short acting ascorbic acid derivative. For example, the cell culture media may contain at least about 0.02 g/L of a short acting ascorbic acid derivative. In another example, the cell culture media may contain at least about 0.03 g/L of a short acting ascorbic acid derivative. For example, the cell culture media may contain at least about 0.04 g/L of a short acting ascorbic acid derivative. In another example, the cell culture media may contain at least about 0.05 g/L of a short acting ascorbic acid derivative. In another example, the cell culture media may contain at least about 0.06 g/L of a short acting ascorbic acid derivative.

[197] In another example, the cell culture media contains a short acting ascorbic acid derivative but does not contain a substantial amount of a long acting ascorbic acid derivative. For example, the cell culture media may contain a short acting ascorbic acid derivative but not more than 0.04 g/L of a long acting ascorbic acid derivative. In another example, the cell culture media may contain a short acting ascorbic acid derivative but not more than 0.03 g/L of a long acting ascorbic acid derivative. In another example, the cell culture media may contain a short acting ascorbic acid derivative but not more than 0.02 g/L of a long acting ascorbic acid derivative. In another example, the cell culture

media may contain a short acting ascorbic acid derivative but not more than 0.01 g/L of a long acting ascorbic acid derivative. In another example, the cell culture media may contain a short acting ascorbic acid derivative but not more than 0.005 g/L of a long acting ascorbic acid derivative. In another example, the cell culture media may contain a short acting ascorbic acid derivative but not a long acting ascorbic acid derivative. In another example, the cell culture media contains L-ascorbate sodium salt but does not contain a substantial amount of L-ascorbic acid-2-phosphate.

Other additives

[198] In an example, the cell culture medium contains human derived additives. For example, human serum and human platelet cell lysate can be added to the cell culture media. In other examples, additional factors can be added to the cell culture medium. For example, the cell culture media can be supplemented with one or more stimulatory factors selected from the group consisting of, platelet derived growth factor (PDGF), fibroblast growth factor 2 (FGF2), epidermal growth factor (EGF), epidermal growth factor (EGF), $1\alpha,25$ - dihydroxyvitamin D3 (1,25D), tumor necrosis factor α (TNF- α), interleukin - β (IL- β) and stromal derived factor 1α (SDF- 1α). In another embodiment, cells may also be cultured in the presence of at least one cytokine in an amount adequate to support growth of the cells. In another embodiment, cells can be cultured in the presence of heparin or a derivative thereof.

[199] In the above examples, basal medium such as Alpha MEM or StemSpan™ can be supplemented with the referenced quantity of serum and, in certain examples, other additives. Further examples of suitable culture mediums for culturing stem cells can be found, for example, in WO2016139340.

Angiogenic markers

[200] According to the present disclosure, in certain embodiments, MLPSCs cultured according to certain methods disclosed herein express increased levels of one or more angiogenic markers. In an example, methods of the disclosure relate to selection of culture expanded MLPSCs for use in treatments such as treatment of congenital heart disease. Such methods comprise determining the level(s) of a marker(s) disclosed herein and, selecting for use in treatment MLPSCs that have increased levels of one or more of the marker(s).

[201] Angiogenesis is the physiological process through which new blood vessels form. In pathophysiological events such as ischemia and inflammation, angiogenesis is increased at the site of injury due to the release of growth factors such as vascular

endothelial growth factor (VEGF) and chemokines such as stromal cell–derived factor 1 (SDF-1). SDF-1 α is a pro-angiogenic protein that is known to play role in the migration, recruitment, and retention of endothelial progenitor cells to sites of ischemic injury and contributes to neovascularization. VEGF is considered the most important regulator of blood vessel formation in health and disease and is essential for embryonic vasculogenesis, angiogenesis, as well as being a key mediator of neovascularization in cancer and other diseases. VEGF acts through a family of cognate receptor kinases in endothelial cells to stimulate blood-vessel formation. At the cellular level, VEGF binding to its main receptor kinase-insert-domain-containing receptor (KDR) imitates a complex network of signalling pathways including activation of phospholipase C-gamma, protein kinase C, Ca(2+), ERK (extracellular-signal-regulated protein kinase), Akt, Src, focal adhesion kinase and calcineurin pathways.

[202] Angiogenin is another potent pro-angiogenic factor that regulates angiogenesis and cell proliferation by stimulating basement membrane degradation, endothelial cell penetration, migration and formation of tubular vascular structures. Angiogenin induces angiogenesis after binding to actin on the surface of endothelial cells. Angiogenin is a member of the RNase A superfamily and is encoded by the ANG gene in humans (NCBI Gene ID: 283; GenBank: AAH62698.1). The structure, function and expression pattern of angiogenin is known in the art, along with methods for detection (see, for example Tello-Montoliu et al. *J Thromb Haemost.* 2006 ;4(9):1864-74.). A range of commercially available antibodies directed to human angiogenin can be used to detect the protein in fluids such as serum, plasma, cell culture supernatant (for example, cell conditioned media), and urine using commercially available enzyme linked immunoabsorbance assays (ELISA) kits. Antibody-based detection assays can also be used to measure angiogenin in tissue or cell lysates. Other approaches to measure angiogenin employ the use of human cytokine protein array technology, for example, Luminex assays, where antibody arrays can be used to simultaneously detect angiogenin among multiple additional factors from a variety of sources.

[203] Methods of the disclosure involve measuring the level of pro-angiogenic factors, such as VEGF, angiogenin and/or SDF-1 α , expressed by MLPSCs under culture conditions. In an example, MLPSCs can be culture expanded in culture media according to the methods disclosed herein. Conditioned media from cultured MLPSCs is then isolated (i.e. a sample is obtained from the cell culture) and the amount of expressed angiogenic marker contained therein is measured. The level of angiogenic markers in MLPSC-conditioned media can be measured by standard protein detection methods and/or gene expression methods known in the art. In an example, the level of angiogenic

marker is measured by enzyme-linked immunosorbent assay (ELISA). For example, conditioned media of MLPSCs is obtained and then contacted with anti-VEGF antibody, anti- SDF-1 α antibody, and/or an anti-angiogenin antibody. Extent of antibody binding is used to quantify the level of angiogenic marker in the conditioned media (e.g. ng/L). In this example, the level of angiogenic marker in the conditioned media is a measure of the level of angiogenic marker expressed or secreted by MLPSCs.

[204] In an example, the level of angiogenic marker is measured by a Western blot. In an example, the level of angiogenic marker is measured by a Luminex assay. In an example, the level of angiogenic marker is measured by reverse transcription RT-PCR. For example, the level of angiogenin in conditioned media from cultured MLPSCs is measured by a cytokine protein array, such as a Luminex assay.

[205] In an example, MLPSCs are selected for use in treatment if they express elevated levels of vascular endothelial growth factor (VEGF). In an example, level of VEGF is greater than about 3 ng/ml. In an example, the level of VEGF is greater than between about 3 ng/ml and 4 ng/ml. In an example, level of VEGF is greater than about 3.1 ng/ml. In an example, level of VEGF is greater than about 3.2 ng/ml. In an example, level of VEGF is greater than about 3.3 ng/ml. In an example, level of VEGF is greater than about 3.4 ng/ml. In an example, level of VEGF is greater than about 3.5 ng/ml. In an example, the level of VEGF is between about 3 ng/ml and 4 ng/ml. In an example, the level of VEGF is between about 3.2 and 3.6 ng/ml. In an example, the level of VEGF is about 3.45 ng/ml.

[206] In an example, MLPSCs are selected for use in treatment if they have increased levels of VEGF relative to a population of MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal calf serum. In an example, the level of VEGF is increased by about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60 %, or about 70% relative to a population of MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal calf serum. In an example, the level of VEGF is increased by between about 5% and about 60%. In an example, the level of VEGF is increased by between about 5% and about 40%. In an example, the level of VEGF is increased by about 40%. In an example, the level of VEGF is increased by at least about 5%. In an example, the level of VEGF is increased by at least about 10%.

[207] In an example, MLPSCs are selected for use in treatment if they express elevated levels of angiogenin. In an example, the level of angiogenin is greater than about 1000 pg/ml. In an example, the level of angiogenin is greater than about 1100 pg/ml. In an example, the level of angiogenin is between about 1000 pg/ml and 1200 pg/ml. In an

example, the level of angiogenin is between about 1100 pg/ml and 1150 pg/ml. In an example, the level of angiogenin is about 1114 pg/ml or higher. In an example, the level of angiogenin is greater than about 1200 pg/ml.

[208] In an example, MLPSCs are selected for use in treatment if they have increased levels of angiogenin relative to a population of MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal calf serum. In an example, the level of angiogenin is increased by about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60 %, or about 70% relative to a population of MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal calf serum. In an example, the level of angiogenin is increased by between about 5% and about 60%. In an example, the level of angiogenin is increased by between about 5% and about 40%. In an example, the level of angiogenin is increased by about 40%. In an example, the level of angiogenin is increased by at least about 5%. In an example, the level of angiogenin is increased by at least about 10%.

[209] In an example, MLPSCs are selected for use in treatment if they express elevated levels of stromal derived factor 1 α (SDF-1 α). In an example, the level of SDF-1 α is greater than about 3000 ng/ml. In an example, the level of SDF-1 α is greater than about 3100 ng/ml. In an example, the level of SDF-1 α is greater than about 3200 ng/ml. In an example, the level of SDF-1 α is greater than about 3300 ng/ml. In an example, the level of SDF-1 α is greater than about 3400 ng/ml. In an example, the level of SDF-1 α is greater than about 3500 ng/ml. In an example, the level of SDF-1 α is between about 3000 ng/ml and 3500 ng/ml. In an example, the level of SDF-1 α is between about 3000 ng/ml and 3400 ng/ml. In an example, the level of SDF-1 α is between about 3000 ng/ml and 3300 ng/ml. In an example, the level of SDF-1 α is between about 3100 ng/ml and 3400 ng/ml. In an example, the level of SDF-1 α is between about 3100 ng/ml and 3300 ng/ml.

[210] In an example, MLPSCs are selected for use in treatment if they have increased levels of SDF-1 α relative to a population of MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal calf serum. In an example, the level of SDF-1 α is increased by about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60 %, or about 70% relative to a population of MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal calf serum. In an example, the level of SDF-1 α is increased by between about 5% and about 60%. In an example, the level of SDF-1 α is increased by between about 5% and about 40%. In an example, the level of SDF-1 α is increased by about 40%. In an example, the level of SDF-1 α is increased by at least about 5%. In an example, the level of SDF-1 α is increased by at least about 10%.

[211] In another example, the angiogenic marker is increased angiogenesis. In this example, increased angiogenesis is measured by an in-vitro angiogenesis assay, for example, a quantitative live-cell imaging assay. Briefly, an endothelial cell line (e.g. human umbilical vein endothelial cells (HUVECs), human dermal fibroblasts, human saphenous vein endothelial cells (HSaVECs), human coronary artery endothelial cells (HCAECs), human aortic endothelial cells (HAECs), brain microvascular endothelial cells (BMEC), or any combination thereof) are fluorescently labelled and seeded into a culture plates. The endothelial cells are then simultaneously incubated in the presence or absence of MLPSC-conditioned media and imaged using a live-cell imaging system.

[212] In this example, angiogenesis can be measured by various network morphometric parameters identified and computed by image analysis software as composite of various elements described in Table 1 (Lam et al. Biomaterials 290. (2022) 121826). In an example, the live-cell imaging system is the IncuCyte® Live-Cell Analysis System. Live-cell imaging systems enable the fluorescent identification of cells and visualization of angiogenesis over time by time-lapse image acquisition. Images can be analysed using computer-based image analysis tools. In an example, the image analysis tool is the IncuCyte® Angiogenesis Analysis Software Module. The IncuCyte® Angiogenesis Analysis Software Module measures angiogenic outputs including endothelial network length, endothelial network area and endothelial branch point formation. The skilled person will appreciate that other image analysis applications can be used, for example Image J, CellProfiler. Other examples of live imaging in-vitro angiogenesis assays are disclosed, for example, in Lam et al. Biomaterials 290. (2022). 121826.

Table 1: Network morphometric parameters

Element	Definition
Node	Pixel having at least three neighbours
Junction	Group of nodes forming a bifurcation
Master Junction	Junctions linked with at least three master segments
Extremity	Pixel having only one neighbour
Segment	Binary line linked with two junctions
Master Segment	Binary lines linked with two junctions that are not exclusive to one branch
Branch	Binary line linked with one junction and one extremity
Isolated Element	Binary line connected to two extremities
Mesh	Area enclosed by segments and corresponding junctions

Element	Definition
Number of Extremities	Number of Extremities in image
Number of Nodes	Number of Nodes in image
Number of Junctions	Number of Junctions in image
Number of Master Junctions	Number of Master Junctions in Image
Number of Master Segments	Number of Master Segments in Image
Total Master Segment Length	Sum of all measured Segments in image
Number of Meshes	Number of meshes in image
Total Mesh Area	Sum of mesh areas in image
Number of Pieces	Number of segments, isolated elements, and branches in image
Number of Segments	Number of segments in image
Number of Branches	Number of branches in image
Number of Isolated Segments	Number of Isolated Segments in image
Total Length	Sum of 'Branch', 'Segment', and 'Isolated Segment' lengths of all vessels in image
Total Branching Length	Sum of all 'Branch' and 'Segment' lengths in image
Total Segments Length	Sum of all 'Segment' lengths in image
Total Branches Length	Sum of all 'Branch' lengths in image
Total Isolated Branches Length	Sum of all 'Isolated Element' lengths in image
Branching Interval	Mean distance separating two branches in image
Mesh Index	Mean distance separating two master junctions in image
Mean Mesh Size	Mean of 'Mesh' area in image (unit - pixel)

[213] In an example, angiogenesis is measured by the level of endothelial network formation, endothelial network length, and/or endothelial branch length. In an example, angiogenic potential is measured by the level of endothelial network formation, endothelial network length, and/or endothelial branch length. In an example, the level of endothelial network formation, endothelial network length, and/or endothelial branch length is measured after treating a population of endothelial cells with conditioned media

obtained from the MLPSCs. In an example, the level of endothelial network formation, endothelial network length, and/or endothelial branch length is calculated by the IncuCyte® Angiogenesis Analysis Software Module.

[214] In another example, the endothelial network formation, endothelial network length, and/or endothelial branch length can be calculated as a composite of one or more of number of nodes, number of junctions, number of segments, number of meshes, mean mesh size, total mesh area, number of extremities, total branches length, and/or number of branches. As used herein, “network formation” refers to the network area in units of mm^2/mm^2 . Further examples of how endothelial network formation, endothelial network length, and/or endothelial branch length can be calculated are described, for example, in Lam et al. *Biomaterials* 290. (2022).

[215] In an example, MLPSCs are selected for use in treatment if they increase the level of one or more of endothelial network formation, endothelial network length, and/or endothelial branch length. In an example, MLPSCs are selected when endothelial network formation is greater than about $0.1 \text{ mm}^2/\text{mm}^2$. In an example, the endothelial network formation is between about $0.1 \text{ mm}^2/\text{mm}^2$ and $0.2 \text{ mm}^2/\text{mm}^2$. In an example, the endothelial network formation is about $0.12 \text{ mm}^2/\text{mm}^2$. In an example, the endothelial network formation is greater than about $0.12 \text{ mm}^2/\text{mm}^2$. In an example, the endothelial network length is greater than about $4 \text{ mm}^2/\text{mm}^2$. In an example, the endothelial network length is between about $4 \text{ mm}^2/\text{mm}^2$ and about $6 \text{ mm}^2/\text{mm}^2$. In an example, the endothelial network length is about $5 \text{ mm}^2/\text{mm}^2$. In an example, the endothelial network length is greater than about $5 \text{ mm}^2/\text{mm}^2$. In an example, the endothelial branch length is greater than about $12 \text{ } 1/\text{mm}^2$. In an example, the endothelial branch length is between about $12 \text{ } 1/\text{mm}^2$ and about $17 \text{ } 1/\text{mm}^2$. In an example, the endothelial branch length is about $15 \text{ } 1/\text{mm}^2$. In an example, the endothelial branch length is greater than about $15 \text{ } 1/\text{mm}^2$.

[216] In an example, MLPSCs are selected for use in treatment if they increase the level of one or more of endothelial network formation, endothelial network length, and/or endothelial branch length relative to a population of MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal calf serum. In an example, the level of endothelial network formation is increased by about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60 %, or about 70% relative to a population of MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal calf serum. In an example, the level of endothelial network formation is increased by between about 5% and about 60%. In an example, the level of endothelial network formation is increased by between about 5% and about 40%. In an example, the level of

endothelial network formation is increased by about 40%. In an example, the level of endothelial network formation is increased by at least about 5%. In an example, the level of endothelial network formation is increased by at least about 10%.

[217] In an example, MLPSCs are selected when the level of endothelial network length is increased by about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60 %, or about 70% relative to a population of MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal calf serum. In an example, the level of endothelial network length is increased by between about 5% and about 60%. In an example, the level of endothelial network length is increased by between about 5% and about 40%. In an example, the level of endothelial network length is increased by about 40%. In an example, the level of endothelial network length is increased by at least about 5%. In an example, the level of endothelial network length is increased by at least about 10%.

In an example, MLPSCs are selected when the level of endothelial branch length is increased by about 5%, about 10%, about 20%, about 30%, about 40%, about 50%, about 60 %, or about 70% relative to a population of MLPSCs that have been culture expanded in a cell culture medium comprising 10% fetal calf serum. In an example, the level of endothelial branch length is increased by between about 5% and about 60%. In an example, the level of endothelial branch length is increased by between about 5% and about 40%. In an example, the level of endothelial branch length is increased by about 40%. In an example, the level of endothelial branch length is increased by at least about 5%. In an example, the level of endothelial branch length is increased by at least about 10%.

Compositions

[218] MLPSCs disclosed herein can be culture expanded from a cryopreserved intermediate to produce a preparation containing at least one therapeutic dose.

[219] In an example, compositions of the disclosure comprise around 150 million cells. In an example, compositions of the disclosure comprise around 20 million cells.

[220] In one example, compositions of the disclosure comprise a pharmaceutically acceptable carrier and/or excipient. The terms "carrier" and "excipient" refer to compositions of matter that are conventionally used in the art to facilitate the storage, administration, and/or the biological activity of an active compound (see, e.g., Remington's Pharmaceutical Sciences, 16th Ed., Mac Publishing Company (1980). A carrier may also reduce any undesirable side effects of the active compound. A suitable carrier is, for example, stable, e.g., incapable of reacting with other ingredients in the

carrier. In one example, the carrier does not produce significant local or systemic adverse effect in recipients at the dosages and concentrations employed for treatment.

[221] Suitable carriers for the present disclosure include those conventionally used, e.g., water, saline, aqueous dextrose, lactose, Ringer's solution, a buffered solution, hyaluronan and glycols are exemplary liquid carriers, particularly (when isotonic) for solutions. Suitable pharmaceutical carriers and excipients include starch, cellulose, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium stearate, glycerol monostearate, sodium chloride, glycerol, propylene glycol, water, ethanol, and the like.

[222] In another example, a carrier is a media composition, e.g., in which a cell is grown or suspended. Such a media composition does not induce any adverse effects in a subject to whom it is administered. Exemplary carriers and excipients do not adversely affect the viability of a cell and/or the ability of a cell to treat or prevent disease.

[223] In one example, the carrier or excipient provides a buffering activity to maintain the cells and/or soluble factors at a suitable pH to thereby exert a biological activity, e.g., the carrier or excipient is phosphate buffered saline (PBS). PBS represents an attractive carrier or excipient because it interacts with cells and factors minimally and permits rapid release of the cells and factors, in such a case, the composition of the disclosure may be produced as a liquid for direct application to the blood stream or into a tissue or a region surrounding or adjacent to a tissue, e.g., by injection.

[224] Compositions of the disclosure may be cryopreserved. Cryopreservation of MLPSCs can be carried out using slow-rate cooling methods or 'fast' freezing protocols known in the art. Preferably, the method of cryopreservation maintains similar phenotypes, cell surface markers and growth rates of cryopreserved cells in comparison with unfrozen cells.

[225] The cryopreserved composition may comprise a cryopreservation solution. The pH of the cryopreservation solution is typically 6.5 to 8, preferably 7.4.

[226] The cyropreservation solution may comprise a sterile, non-pyrogenic isotonic solution such as, for example, PlasmaLyte ATM. 100 mL of PlasmaLyte ATM contains 526 mg of sodium chloride, USP (NaCl); 502 mg of sodium gluconate (C₆H₁₁NaO₇); 368 mg of sodium acetate trihydrate, USP (C₂H₃NaO₂•3H₂O); 37 mg of potassium chloride, USP (KCl); and 30 mg of magnesium chloride, USP (MgCl₂•6H₂O). It contains no antimicrobial agents. The pH is adjusted with sodium hydroxide. The pH is 7.4 (6.5 to 8.0).

[227] The cryopreservation solution may comprise ProfreezeTM. The cryopreservation solution may additionally or alternatively comprise culture medium, for example, α MEM.

[228] To facilitate freezing, a cryoprotectant such as, for example, dimethylsulfoxide (DMSO), is usually added to the cryopreservation solution. Ideally, the cryoprotectant should be nontoxic for cells and patients, nonantigenic, chemically inert, provide high survival rate after thawing and allow transplantation without washing. However, the most commonly used cryoprotector, DMSO, shows some cytotoxicity. Hydroxyethyl starch (HES) may be used as a substitute or in combination with DMSO to reduce cytotoxicity of the cryopreservation solution.

[229] The cryopreservation solution may comprise one or more of DMSO, hydroxyethyl starch, human serum components and other protein bulking agents. In one example, the cryopreserved solution comprises Plasma-Lyte A (70%), DMSO (10%), HSA (25%) solution, the HSA solution comprising 5% HSA and 15% buffer.

[230] In an example, the cryopreservation solution may further comprise one or more of methycellulose, polyvinyl pyrrolidone (PVP) and trehalose.

[231] The cryopreserved composition may be thawed and administered directly to the subject or added to another solution, for example, comprising hyaluronic acid. Alternatively, the cryopreserved composition may be thawed and the MLPSCs resuspended in an alternate carrier prior to administration.

[232] The compositions described herein may be administered alone or as admixtures with other cells. The cells of different types may be admixed with a composition of the disclosure immediately or shortly prior to administration, or they may be co-cultured together for a period of time prior to administration.

[233] In one example, the composition comprises an effective amount or a therapeutically or prophylactically effective amount of MLPSCs and/or progeny thereof and/or soluble factor derived therefrom. The exact amount of cells to be administered is dependent upon a variety of factors, including the age, weight, and sex of the subject, and the extent and severity of the disorder being treated.

[234] Despite the number of cells provided in the composition, in an example, 20×10^6 are administered.

[235] In an example, the composition comprises greater than 5.00×10^6 viable cells/mL. In another example, the composition comprises greater than 5.50×10^6 viable cells/mL. In another example, the composition comprises greater than 6.00×10^6 viable cells/mL. In another example, the composition comprises greater than 6.50×10^6 viable

cells/mL. In another example, the composition comprises greater than 6.68×10^6 viable cells/mL.

[236] In an example, the MLPSCs comprise at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 99% of the cell population of the composition.

[237] In an example, the composition may optionally be packaged in a suitable container with written instructions for a desired purpose.

[238] In an example, compositions are administered transendocardially. In an example, compositions are administered to the heart wall of the left ventricle. In an example, the composition is administered to the myocardium. In an example, the composition is administered to the LV endocardium. In another example, the composition is administered over 7 – 15 injections to the LV endocardium. In an example, at least two of the injections are administered to the two LV papillary muscles. In an example, at least two of the injections are administered to the two LV papillary muscles and the remainder of the injections are administered over the upper, mid, and apical regions of the LV endocardium.

[239] In an example, compositions of the disclosure comprise a “clinically proven effective” amount of MLPSCs. In an example, compositions of the disclosure comprise a “clinically proven effective” amount of MSCs. In an example, compositions of the disclosure comprise a “clinically proven effective” amount of MPCs.

[240] In an example, the “clinically proven effective” amount of MLPSCs is administered as a total dose. The term “total dose” is used in the context of the present disclosure to refer to the total number of cells received by the subject treated according to the present disclosure. In an example, the total dose consists of one administration of cells. In another example, the total dose consists of two administrations of cells. In another example, the total dose consists of three administrations of cells. In another example, the total dose consists of four or more administrations of cells. For example, the total dose can consist of two to four administrations of cells.

Methods of treatment

[241] Methods of the present disclosure relate to treating congenital heart disease in a subject, the method comprising administering to the subject a composition comprising a MLPSC population disclosed herein and/or conditioned media obtained therefrom.

Accordingly, in an example, methods of the disclosure comprise administering culture expanded MLPSCs. In another example, methods of the disclosure comprise administering conditioned media or soluble factors obtained therefrom.

[242] The term “congenital heart disease (CHD)” is used in the context of the present disclosure to refer to abnormality in the structure or function of the cardio-circulatory system present at birth. Examples of congenital heart disease include ventricular septal defect (VSD), atrial septal defect (ASD), patent ductus arteriosus (PDA), AV septal defect (AVSD), coarctation of aorta (CoA), aortic stenosis (AS) bicuspid aortic valve (BAV), tetralogy of Fallot (TOF), transposition of great arteries (TGA), tricuspid atresia (TA), total/partial anomalous pulmonary venous connection (T/PAPVC), persistent truncus arteriosus (PTA), Ebstein’s anomaly (EA), double outlet right ventricle (DORV), pulmonary stenosis (PS).

[243] In an example, congenital heart disease treated according to the present disclosure comprises a spectrum of congenital heart malformations. For example, the congenital heart disease according to the present disclosure is hyperplastic left heart syndrome (HLHS). HLHS is a spectrum of severe congenital heart disease (CHD) characterised by underdeveloped left-sided cardiac structures. Features of HLHS include mitral valve and aortic valve atresia or stenosis, with resultant hypoplasia or absence of the left ventricle and hypoplasia of the ascending aorta and aortic arch.

[244] In an example, the present disclosure relates to the treatment of subjects with congenital heart disease, such as HLHS, and persistent inflammation. “Persistent inflammation” is defined by elevated C-reactive protein levels. In an example, persistent inflammation is characterised by CRP levels ≥ 2 mg/L. Accordingly, in this example, the present disclosure relates to the treatment of subjects with HLHS and CRP levels ≥ 2 mg/L.

[245] In an example, subjects treated according to the present disclosure have an initial CRP level < 5 mg/L. In another example, subjects have an initial CRP level < 4 mg/L. In another example, subjects have an initial CRP level between 2 and 6 mg/L. In another example, subjects have an initial CRP level between 3 and 6 mg/L. In another example, subjects have an initial CRP level between 4 and 5 mg/L.

[246] There are various assays available for measuring CRP levels such as antibody based immunoassays. For example, CRP levels can be measured in blood samples using an Enzyme-Linked Immunosorbent (ELISA) assay. In an example, a blood sample is obtained from a patient and then purified before being contacted with anti-CRP antibody. Extent of antibody binding is used to quantify the level of CRP in the blood sample (e.g. mg/L).

[247] In an example, subjects treated according to the present disclosure have undergone single ventricle palliation. For example, subjects treated according to the disclosure have undergone a Norwood procedure.

[248] In another example, subjects treated according to the disclosure have undergone a bidirectional Glenn surgery (i.e. superior cavopulmonary anastomosis). In another example, subjects are being undergoing a bidirectional Glenn surgery at the time of being treated with a MLPSC composition disclosed herein.

[249] In an example, methods of the present disclosure reduce or inhibit progression of the congenital heart disease. In an example, treatment improves the subject's left ventricular function. For example, the subject's left ventricular function is improved by increasing one or more of left ventricular end diastolic volume (LVEDV), left ventricular end systolic volume (LVESV), LV stroke volume, left ventricular end diastolic pressure (LVEDP). Accordingly, the present disclosure also provides a method of improving left ventricular function in a subject suffering from congenital heart disease comprising administering a MLPSC composition disclosed herein in an amount effective to increase LVEDV, left ventricular end systolic LVESV, LV stroke volume, and/or LVEDP.). In an example, treatment also preserves left ventricular ejection fraction (LVEF). In an example, LVEF is preserved for at least 12 months after administration of MLPSCs.

[250] As used herein, the term "left ventricular function" refers to the ability of the left ventricle to pump blood through the body with each heartbeat. Left ventricular function can be assessed by measuring by one or more parameters such as LVESV, LVEDV, LVEF, LV stroke volume, and LVEDP. As the person skilled in the art will appreciate, LVEDV is the volume of blood in the left ventricle at the end of diastole (before contraction) and LVESV refers to the volume of blood in the left ventricle at the end of systole (after contraction). LVEF, expressed as a percentage, is calculated using the following formula: $(LVEDV - LVESV) / LVEDV$.

[251] In an example, treatment increases a subject's LVESV. In an example, treatment increases the subject's LVESV by at least 15 ml/m². In another example, treatment increases the subject's LVESV by at least 20 ml/m². In another example, treatment increases the subject's LVESV by at least 25 ml/m². In another example, treatment increases the subject's LVESV between 20 ml/m² and 50 ml/m². In an example, the increase in a subject's LVESV from baseline is observed 12 months after administering a composition disclosed herein.

[252] In an example, treatment increases a subject's LVEDV. In an example, treatment increases the subject's LVEDV by at least 50 ml/m². In another example, treatment increases the subject's LVEDV by at least 65 ml/m². In another example,

treatment increases the subject's LVEDV by at least 80 ml/m². In another example, treatment increases the subject's LVEDV between 60 ml/m² and 120 ml/m². In an example, the increase in a subject's LVEDV from baseline is observed 12 months after administering a composition disclosed herein.

[253] In an example, the increase in a subject's LVESV and/or LVEDV is measured by 3-D echocardiography. In example, a treated subject's LVESV and/or LVEDV is increased relative to a subject with a comparable congenital heart disease that has not been administered MLPSCs.

[254] In example, treatment increases the subject's LVEF. In an example, the increase in a subject's LVEF from baseline is observed 12 months after administering a composition disclosed herein.

[255] In an example, treatment increases a subject's left ventricular stroke volume. In an example, LV stroke volume is increased by at least 10 ml/m². In another example, treatment increases the subject's LV stroke volume by at least 15 ml/m². In another example, treatment increases the subject's LV stroke volume by at least 20 ml/m². In another example, treatment increases the subjects LV stroke volume between 10 ml/m² and 30 ml/m². In an example, the increase in a subject's LV stroke volume from baseline is observed 12 months after administering a composition disclosed herein. In an example, the increase in a subject's LV stroke volume is determined by 3D-echocardiography.

[256] In an example, treatment increases a subject's LVEDP. LVEDP provides a measure of preload, which is the amount of stretch on the myocardial muscle fibers just before contraction. In an example, treatment increases a subject's LVEDP by at least 1 mmHg. In an example, treatment increases a subject's LVEDP by at least 2 mmHg. In an example, treatment increases a subject's LVEDP by at least 3 mmHg. In an example, treatment increases a subject's LVEDP by at least 4 mmHg. In an example, treatment increases a subject's LVEDP by at least 5 mmHg. In an example, the increase in a subject's LVEDP is observed 12 months after administering a composition disclosed herein. In an example, LVEDP is measured by cardiac catheterisation.

Adjuvant therapy

[257] The term "adjuvant therapy" in the context of the present disclosure refers to a treatment that is, given in addition to a primary therapy, such as a definitive left ventricular surgery. As the person skilled in the art will appreciate, an adjuvant therapy may improve a subject's response to the primary therapy, increase the efficacy of the primary therapy, or a combination thereof. In an example, the adjuvant therapy prepares

the subject's heart for the primary therapy. The present inventors have found that administering MLPSCs to subjects with congenital heart disease improves the subject's left ventricular function which, in turn, increases the subject's prospects for successful definitive left ventricular surgery. In one example, the definitive left ventricular surgery is biventricular conversion (BiV). In one example, the definitive left ventricular surgery is a bidirectional Glenn surgery. In one example, the method of adjuvant therapy comprises the following steps:

- (i) selecting a subject having congenital heart disease for treatment;
- (ii) administering MLPSCs to the subject;
- (iii) determining if the subject's cardiac performance is improved; and
- (iv) wherein upon determining that the subject's cardiac performance is improved, performing a definitive left ventricular surgery.

[258] In an example, definitive left ventricular surgery is performed if the subject's LVESV, LVEDV, LVEF, LV stroke volume, and/or LVEDP is increased following administration of MLPSCs. In an example, the subject's LVESV, LVEDV, LVEF, LV stroke volume, and/or LVEDP is increased relative to baseline. In an example, the subject's LVESV, LVEDV, LVEF, LV stroke volume, and/or LVEDP is increased within 12 months following administration of MLPSCs. In an example, definitive left ventricular surgery is performed when LVEDV is increased by at least 50 ml/m², 65 ml/m², 80 ml/m². In an example, definitive left ventricular surgery is performed when LVEDV is increased by between 60 ml/m² and 120 ml/m². In one example, definitive left ventricular surgery is performed when LVESV is increased by at least 15 ml/m², 20 ml/m², or 25 ml/m². In one example, definitive left ventricular surgery is performed when LVESV is increased by between 20 ml/m² and 50 ml/m². In one example, In one example, definitive left ventricular surgery is performed LV stroke volume is increased by at least 5 to 20 ml/m². In one example, definitive left ventricular surgery is performed when LVEDP is increased by at least 5 mmHg.

[259] In an example, definitive left ventricular surgery is performed on the subject within 2 years following administration of MLPSCs. In one example, definitive left ventricular surgery is performed between 12 months and 2 years following administration of MLPSCs. For example, definitive left ventricular surgery is performed 12 months, 14 months, 16 months, 18 months, 20 months, 22 months, or 2 years following administration of MLPSCs. In an example, definitive left ventricular surgery is performed between 12 months and 22 months administration of MLPSCs. In one example, definitive left ventricular surgery is performed between 12 and 18 months following administration of MLPSCs.

Biventricular conversion

[260] In an example, the present disclosure relates to a method of biventricular conversion (BiV) in subjects with a congenital heart disease disclosed herein, such as HLHS. BiV aims to convert a subject's ventricular arrangement into two pumping ventricles. In this example, the BiV is staged, with MLPSCs being administered during single ventricle palliation and/or superior cavopulmonary anastomosis. However, attempts at early biventricular conversion (BiV) may carry higher perioperative risk and lead to long-term diastolic dysfunction in some subjects. In an example, the BiV is performed within 2 years of administering MLPSCs. In an example, the BiV is performed at least 12 months after administering MLPSCs. In an example, the BiV is performed between 12 months and 2 years after administering MLPSCs. In an example, the subject has improved cardiac performance prior to BiV. In an example, the improved cardiac performance is observed relative to a subject with a comparable congenital heart disease that has not been administered MLPSCs. In an example, the subject's likelihood of achieving successful BiV circulation is increased. For example, the subject's likelihood of achieving successful BiV circulation is increased by at least 50% relative to a control subject who was not administered MLPSCs. In an example, the subject achieves successful BiV circulation within 2 years after administration of MLPSCs.

[261] In an example, the present disclosure relates to a method of treating a subject with a congenital heart disease, the method comprising single ventricle palliation, superior cavopulmonary anastomosis, administering a composition comprising a population of culture expanded mesenchymal lineage precursor or stem cells (MLPSCs) or conditioned media obtained therefrom, and performing BiV. In an example, the congenital heart disease is HLHS. In an example, the MLPSCs are administered with single ventricle palliation. In another example, the MLPSCs are administered with superior cavopulmonary anastomosis. In another example, the MLPSCs are administered with single ventricle palliation and superior cavopulmonary anastomosis. In an example, the BiV is performed at least 12 months after administering the MLPSCs.

[262] In some examples, the subject receives a one-and-a-half ventricle repair. Accordingly, in an example, a one-and-a-half ventricle repair (1.5V) is performed on the subject. In an example, a reverse one-and-a-half ventricle repair (1.5V) is performed on the subject.

Selecting a subject for treatment

[263] In an example, the methods of the present disclosure relate to methods of selecting a subject with congenital heart disease for treatment with stem cell compositions according to the disclosure. In an example, subjects with HLHS are selected for treatment. In an example, subjects that have undergone single ventricle palliation are selected for treatment. In an example, subjects that have undergone superior cavopulmonary anastomosis are selected for treatment. In an example, subjects that have undergone single ventricle palliation and superior cavopulmonary anastomosis are selected for treatment. In an example, subjects with persistent inflammation are selected for treatment. For example, subjects with CRP ≥ 2 mg/L are selected for treatment.

[264] Accordingly, in an example, the present disclosure relates to a method of treating a congenital heart disease, the method comprising the steps of: i) selecting a subject having a CRP level ≥ 2 mg/L for treatment, and ii) administering to the subject a composition comprising MLPSCs.

[265] Accordingly, in an example, subjects with HLHS that have undergone superior cavopulmonary anastomosis are selected for treatment. Such subjects may also be selected on the basis of persistent inflammation. In an example, subjects are selected for adjuvant treatment with MLPSCs if they have LV dysfunction. In an example, a subject is selected for treatment if they have decreased LVESV, LVEDV, LVEF, LV stroke volume, and/or LVEDP. As the person skilled in the art will appreciate, the subject has decreased LVESV, LVEDV, LVEF, LV stroke volume, and/or LVEDP relative to a normal range expected for a healthy subject with normal LV function.

[266] In another example, subjects are selected for a definitive left ventricular surgery based on their LV function and/or LV size after administration of MLPSCs disclosed herein.

EXAMPLES

Patients

[267] Nineteen patients were randomized for this trial. Nine patients were randomized to Mesenchymal lineage cells (MPCs) injections and ten patients were randomized as controls (standard of care surgical treatment with no injections).

[268] Demographic and select baseline clinical information for the trial participants are shown in Table 1 and were similar between the MPC and control groups. 15/19 patients (79%) had HLHS, while 4/19 patients (21%) had unbalanced atrioventricular (AV) canal. Three patients had undergone prior LV recruitment procedures (e.g., EFE resection) during prior surgeries. The median age at randomization was 0.9 years (range

0.3-3.2 years), 5/19 (26%) of patients were female. At baseline, median LV end diastolic volume (LVEDV) indexed to body surface area (BSA) using 3-D echocardiography was 19.9 ml/m² (IQR 15.1 – 28.3 ml/m²) and the median LV mass indexed to BSA was 19.1 g/m² (IQR 17.2 – 25.9 g/m²).

Table 1: Demographic Information and Select Baseline Clinical Characteristics, by Treatment Assigned

	Total (n=19)	MPC (n=9)	Control (n=10)
Age at randomization (months), [IQR]	11.5 [5.8, 28.4]	17.9 [6.1, 27.0]	9.0 [4.6, 28.4]
Sex female	5 (26%)	3 (33%)	2 (20%)
Race/ethnicity			
White	18 (95%)	8 (89%)	10 (100%)
Hispanic	1 (5%)	1 (11%)	0 (0%)
Cardiac diagnosis			
HLHS/HLHS Variant	15 (53%)	7 (56%)	8 (50%)
Unbalanced AVC	4 (21%)	2 (22%)	2 (20%)
Any past cardiac surgery	19 (100%)	9 (100%)	10 (100%)
Underwent LV recruitment surgery prior to trial enrolment	3 (16%)	1 (11%)	2 (20%)
Bidirectional Glenn surgery prior to trial enrolment	10 (53%)	5 (56%)	5 (50%)
Weight (kg), [IQR]	8.7 [6.4, 12.6]	8.7 [6.5, 12.6]	8.6 [6.4, 11.7]
Height (cm), [IQR]	74 [62, 88]	74 [65, 88]	71 [62, 88]
LV end diastolic volume indexed to BSA (mL/m ²) by 3-D Echocardiography, [IQR]	19.9 [15.1, 28.3]	17.4 [14.1, 21.9]	27.1 [16.4, 33.8]
LV mass indexed to BSA (g/m ²) by 3-D Echocardiography, [IQR]	19.1 [17.2, 25.9]	18.5 [16.5, 24.0]	20.6 [18.2, 29.0]

MPC administration

[269] Proprietary STRO-3 selected allogenic mesenchymal lineage stem cells derived from the bone marrow of three young, healthy adult donors and expanded ex vivo were administered. A low dose of approximately 20 million MPCs was chosen based on previous adult studies, proportional to LV mass in HLHS patients. MPCs were delivered to the LV endocardium under direct surgical visualization with a 23-25 gauge needle,

using approximately 11 injections of 50µl each. One injection was performed into each of the two LV papillary muscles, with the remaining nine injections distributed throughout the upper, mid, and apical regions of the LV endocardium to ensure diffuse distribution throughout the LV.

Human Leukocyte Antigen-Sensitization

[270] Both the MPC and control groups had a bimodal PRA distribution, with overall high human leukocyte antigen (HLA)-sensitization. At baseline, 11/18 (61%) of patients had PRA>10% and 9/18 (50%) had PRA>90% with no difference in PRA% between groups across all timepoints ($p=0.73$). The majority of subjects maintained relatively constant PRA levels from baseline through end of follow-up.

Biventricular and 1.5 Ventricle Conversion

[271] Overall, 9 patients (47%) underwent complete BiV conversion and 3 patients (16%) underwent 1.5V conversion prior to final two-year follow-up. Six patients (32%) remained on a single ventricle palliation strategy at two-year follow-up and one patient (5%) had a single ventricle palliation strategy but had not yet completed follow-up. No patients proceeded to Fontan palliation prior to two-year follow-up. There were no significant differences between the MPC and control groups in time to BiV/1.5V conversion ($p=0.86$). In the overall cohort, the probability of BiV/1.5V conversion was 0.16 (95% CI 0.05-0.41) at 12 months and 0.52 (95% CI 0.31-0.77) at 24 months (Figure 2). For MPC-treated children, 100% (5 of 5) were able to undergo the preferred full BiV conversion. No MPC-treated patients underwent the 1.5V surgical procedure. In contrast, for the control children, 57% (4 of 7) had the full BiV conversion whereas 43% underwent the more limited 1.5V surgery.

Imaging and Catheterization Data

[272] Baseline data were similar between the MPC and control groups for all imaging modalities. There was a significant increase in LVEDV, LVESV, and LV Mass from baseline to 12 months in both the MPC and control groups. By 3-D echocardiography, the MPC group had a significantly larger increase in LVEDV ($p=0.020$) and LVESV ($p=0.009$) indexed to BSA from baseline to 12 months, compared to the control group. At 12 months, median LVEDV in the MPC group was 81.7 mL/m² (IQR 56.7–107.8 mL/m²) compared to 41.8 mL/m² (37.3–60.8 mL/m²) in the control group. Median LVESV in the MPC group was 28.1 mL/m² (IQR 20.8–40.8 mL/m²) compared to 12.9 mL/m² (IQR 11.3–23.9 mL/m²) in the control group. LV mass indexed to BSA and LV

EF significantly increased from baseline to 12 months in the overall cohort but there was no significant difference between groups ($p=0.85$ and $p=0.34$, respectively).

[273] On assessment with cardiac catheterization, median change in LVEDP indexed to BSA from baseline to 12 months was 5 mmHg in the MPC group and 0 mmHg in the control group ($p=0.006$).

[274] These imaging data are represented in Table 2 and Figure 3A-C.

Table 2: LV Size and Function from Baseline to 12 Months by 3-D Echocardiography and Cardiac Catheterization, Overall Cohort

	n	Total (n=19)	MPC (n=9)	Control (n=10)	P Value
3-D Echocardiography					
LV end diastolic volume indexed to BSA (mL/m ²)					
Baseline	14, 8, 6	19.9 [15.1, 28.3]	17.4 [14.1, 21.9]	27.1 [16.4, 33.8]	0.020
3 weeks	6, 4, 2	24.9 [20.5, 29.6]	29.2 [24.7, 35.5]	16.3 [11.7, 20.9]	
3-6 months	2, 1, 1	30.3, 37.2	37.2	30.3	
12 months	9, 3, 6	56.7 [41.4, 81.7]	81.7 [56.7, 107.8]	41.8 [37.3, 60.8]	
LV end systolic volume indexed to BSA (mL/m ²)					
Baseline	14, 8, 6	7.8 [7.0, 12.4]	7.6 [6.5, 8.5]	14.0 [8.0, 17.1]	0.009
3 weeks	6, 4, 2	8.2 [7.0, 11.1]	9.8 [8.2, 13.9]	5.5 [4.0, 7.0]	
3-6 months	2, 1, 1	10.6, 13.8	13.8	10.6	
12 months	9, 3, 6	20.8 [12.3, 28.1]	28.1 [20.8, 40.8]	12.9 [11.3, 23.9]	
LV mass indexed to BSA (g/m ²)					
Baseline	14, 8, 6	19.1 [17.2, 25.9]	18.5 [16.5, 24.0]	20.6 [18.2, 29.0]	0.85
3 weeks	6, 4, 2	21.0 [16.4, 27.5]	25.5 [21.0, 32.1]	13.7, 16.4	
3-6 months	2, 1, 1	19.5, 35.2	35.2	19.5	
12 months	8, 2, 6	32.4 [26.4, 49.1]	28.3, 64.4	32.4 [24.6, 43.5]	
LV ejection fraction (%)					
Baseline	14, 8, 6	56.0 [50.8, 61.4]	58.7 [49.3, 64.6]	52.6 [50.8, 58.0]	0.34
3 weeks	7, 5, 2	66.3 [61.8, 67.7]	63.0 [61.8, 67.7]	66.3, 67.0	
3-6 months	2, 1, 1	62.8, 65.0	62.8	65.0	
12 months	9, 3, 6	65.6 [62.2, 67.8]	63.0 [62.2, 65.6]	67.4 [60.7, 69.7]	

Cardiac Catheterization					
LV end diastolic pressure (mmHg)					
Baseline	17, 8, 9	12 [9, 13]	9 [6.5, 13.5]	12 [11, 13]	0.006
12 months	15, 7, 8	13 [10, 16]	16 [10, 18]	12 [8.5, 15.5]	
Difference	14, 7, 7	3 [0, 5]	5 [4, 8]	0 [-3, 3]	
Mean LA pressure (mmHg)					
Baseline	18, 9, 9	8.5 [8, 9]	8 [8, 9]	9 [8, 9]	0.68
12 months	16, 8, 8	14 [12.5, 15]	14 [12, 15]	13.5 [12.5, 14.5]	
Difference	15, 8, 7	5 [4, 7]	5.5 [3.5, 7.5]	5 [4, 6]	

Summary of results

[275] MPC injection in the hypoplastic LV was a safe and feasible adjunctive therapy to surgical LV recruitment. There were no serious adverse events (SAEs) deemed related to the trial and no difference in the frequency, timing, or severity of adverse events (AEs) between groups. There was also no minimal evidence of immunological reaction to the MPC injections, including donor antigen sensitization, local tissue inflammation, arrhythmia, or tumor formation.

[276] Preliminary analyses of LV size and function demonstrated a larger increase in LVEDV and LVESV in the MPC-treated group at one year of follow-up. Without wishing to be bound by any particular theory, these promising results suggest potentially greater LV growth and remodeling in patients receiving MPCs. Additionally, without wishing to be bound by any particular theory, the fact that 100% of MPC-treated children compared with 57% of controls had large enough LVs to accommodate the full BiV conversion suggest that MPC treatment may help to better grow the HLHS LV after LV recruitment surgery.

[277] At 12 months follow-up, estimated LV stroke volume in MPC treated patients was approximately 100% larger than for controls as measured by both 3D-echocardiography (37.7 mL/m² [MPC] vs. 20.2 mL/m² [control]). This sub-analysis suggests that MPC treated patients may have increased LV chamber remodeling at 12 months compared to controls, a finding that could translate into improved cardiac output and systemic perfusion over time (Figure 4).

[278] Our study used allogenic bone marrow-derived mesenchymal stem cells, which have been shown to be equally safe as autologous stem cells, with potentially increased efficacy and have been studied in pediatric populations with HLHS.

[279] The high rate of BiV in children who received MLPSCs into the left ventricle, supports the potential for using MLPSCs as adjuvant therapy for definitive left ventricular surgical procedures to avoid the need for Fontan surgery.

[280] It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the disclosure as shown in the specific embodiments without departing from the spirit or scope of the disclosure as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

[281] The present application claims priority from US63/584,715 filed 22 September 2023, the disclosures of which are incorporated herein by reference.

[282] All publications discussed and/or referenced herein are incorporated herein in their entirety.

[283] Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present disclosure. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each claim of this application.

CLAIMS

1. A method of treating congenital heart disease in a subject, the method comprising administering to the subject a composition comprising a population of culture expanded mesenchymal lineage precursor or stem cells (MLPSCs) or conditioned media obtained therefrom.
2. The method of claim 1, wherein the subject has hypoplastic left heart syndrome.
3. The method of claim 1 or claim 2, wherein the MLPSCs are allogenic.
4. The method according to any one of claims 1 to 3, wherein the composition is administered to the heart wall of the left ventricle.
5. The method according to any one of claims 1 to 4, wherein treatment increases left ventricular end diastolic volume (LVEDV) and/or left ventricular end systolic volume (LVESV) from baseline 12 months after administering the composition.
6. The method according to claim 5, wherein LVEDV and/or LVESV is increased relative to a subject with a comparable congenital heart disease that has not been administered MLPSCs.
7. The method according to any one of claims 1 to 6, wherein treatment increases left ventricular end diastolic pressure (LVEDP) from baseline 12 months after administering the composition, preferably by at least 5 mmHg.
8. The method according to any one of claims 1 to 7, wherein treatment increases LV stroke volume, preferably wherein LV stroke volume is increased by at least 5 to 20 ml/m².
9. The method according to any one of claims 1 to 8, wherein the MLPSCs in the composition have been culture expanded in a cell culture media comprising at least one pro-inflammatory cytokine.

10. The method of claim 9, wherein the media contains serum which comprises the pro-inflammatory cytokine(s), preferably wherein the serum is newborn mammalian serum.
11. The method according to claim 9 or claim 10, wherein the media comprises a non-fetal serum.
12. The method according to claim 10 or 11, wherein the serum is newborn calf serum (NBCS).
13. The method according to any one of claims 10 to 12, wherein the serum is obtained no more than 21 days after birth.
14. The method according to any one of claims 10 to 13, wherein the serum is obtained between the day of birth and 10 days after birth.
15. The method according to any one of claims 10 to 14, wherein the media comprises at least 5% (v/v) newborn calf serum.
16. The method according to any one of claims 1 to 15, wherein the MLPSCs are mesenchymal precursor cells (MPCs).
17. The method according to any one of claims 1 to 15, wherein the MLPSCs are MPCs that are isolated from bone mononuclear cells with an anti-STRO-3 antibody before culture expansion.
18. The method according to any one of claims 1 to 15, wherein the MLPSCs are mesenchymal stem cells (MSCs).
19. The method according to any one of claims 1 to 18, wherein the MLPSCs have been cryopreserved prior to administration.
20. The method according to any one of claims 1 to 19, wherein the composition further comprises Plasma-Lyte A, dimethyl sulfoxide (DMSO), human serum albumin (HSA).

21. The method according to any one of claims 1 to 20, wherein the composition comprises greater than 6.68×10^6 viable cells/mL.
22. The method according to any one of claims 1 to 16, 20 or 21, wherein the composition comprises human bone marrow-derived allogeneic MPCs isolated from bone mononuclear cells with anti-STRO-3 antibodies, expanded *ex vivo* in culture media comprising NBCS, and cryopreserved.
23. The method according to any one of claims 1 to 22, wherein the MLPSCs in the composition are characterised by one or more of the following:
- the MLPSCs express a level of angiogenin greater than about 1200 pg/ml under culture conditions;
 - conditioned media obtained from the MLPSCs under culture conditions induce endothelial network formation greater than about $0.12 \text{ mm}^2/\text{mm}^2$;
 - conditioned media obtained from the MLPSCs under culture conditions induce endothelial network length greater than about $5 \text{ mm}^2/\text{mm}^2$;
 - conditioned media obtained from the MLPSCs under culture conditions induce endothelial branch length greater than about 15 1/mm^2 .
24. A method of adjuvant therapy comprising administering to a subject a composition comprising a population of culture expanded mesenchymal lineage precursor or stem cells (MLPSCs) or conditioned media obtained therefrom, wherein the subject has a congenital heart disease, and wherein the composition is administered as an adjuvant therapy prior to definitive left ventricular surgery.
25. The method of claim 24, wherein the subject has hypoplastic left heart syndrome.
26. The method of claim 24 or claim 25, wherein the definitive left ventricular surgery is a bidirectional Glenn surgery.
27. The method according to any one of claims 24 to 26, wherein the definitive left ventricular surgery is performed within 2 years of administering the composition.
28. The method according to any one of claims 24 to 27, wherein the definitive left ventricular surgery is performed at least 12 months after administering the composition.

29. A method of improving left ventricular function in a subject suffering from congenital heart disease, the method comprising administering to the subject a composition comprising a population of culture expanded mesenchymal lineage precursor or stem cells (MLPSCs) or conditioned media obtained therefrom in an amount effective to increase left ventricular end diastolic volume (LVEDV), left ventricular end systolic volume (LVESV), LV stroke volume, and/or left ventricular end diastolic pressure (LVEDP).

30. The method of claim 29, wherein LVEDV, LVESV, LV stroke volume, and/or LVEDP is increased from baseline 12 months after administering the composition.

31. The method according to claim 29 or 30, wherein

- LVEDV is increased by at least 50 ml/m², preferably 65 ml/m², preferably 80 ml/m², preferably between 60 ml/m² and 120 ml/m²;
- LVESV is increased by at least 15 ml/m², preferably 20 ml/m², preferably 25 ml/m², preferably between 20 ml/m² and 50 ml/m²;
- LV stroke volume is increased by at least 5 to 20 ml/m²; and/or
- LVEDP is increased by at least 5 mmHg.

32. The method according to any one of claims 1 to 31, wherein the MLPSCs are culture expanded from a population of cells which comprise about 0.1% to 75% STRO-1+ cells.

33. The method of claim 32, wherein the population of cells which comprise about 0.1% to 75% STRO-1+ cells are isolated using a STRO-3 antibody.