MESENCHYMAL STEM CELL PARTICLES

FIELD

The present invention relates to the fields of development, cell biology, molecular biology and genetics. More particularly, the invention relates to a method of deriving mesenchymal stem cells from embryonic stem cells.

BACKGROUND

Stem cells, unlike differentiated cells, have the capacity to divide and either self-renew or differentiate into phenotypically and functionally different daughter cells (Keller, Genes Dev. 2005;19:1129-1155; Wobus and Boheler, *Physiol Rev*. 2005;85:635-678; Wiles, *Methods in Enzymology*. 1993;225:900-918; Choi et al, *Methods Mol Med*. 2005;105:359-368).

Mesenchymal stem cells (MSCs) are multipotent stem cells that have documented evidence of therapeutic efficacy in treating musculoskeletal injuries, improving cardiac function in cardiovascular disease and ameliorating the severity of GVHD (Le Blanc and Pittenger, 2005). Being lineage restricted, they have limited but robust potential to differentiate into mesenchymal cell types, e.g adipocytes, chondrocytes and osteocytes, and have negligible risk of teratoma formation. Host immune rejection of transplanted MSCs is routinely circumvented through autologous or allogeneic transplantation. MSCs can be isolated from several adult tissues including bone marrow (BM), adipose tissues (ad), cord blood and expanded *ex vivo*.

Mesenchymal stem cells have been used in clinical and preclinical applications to treat a wide range of diseases[1, 2] including musculoskeletal tissue bioengineering[3, 4] and heart disease[5, 6]. The therapeutic capacity of MSCs to treat a wide spectrum of diseases in clinical and preclinical applications to treat a wide range of diseases [A1,A2] e.g. GVHD [A1] in musculoskeletal tissue bioengineering [A3,A4] and heart disease [A5,A6] has been attributed to their potential to differentiate into many different reparative cell types.

However, availability of tissues for their isolation remains limiting and requires risky invasive procedures, and *ex vivo* expansion of MSCs while significant, is nonetheless finite. However, the efficiency of transplanted MSCs to differentiate into functional reparative cells in the injured tissues or organs, and in therapeutically relevant numbers have never been adequately documented or demonstrated.

Recent reports have suggested that some of these reparative effects may be mediated by paracrine factors secreted by the MSCs[7]. These factors are postulated to promote arteriogenesis through paracrine mechanisms[8], support the stem cell crypt in the intestine[9], protect against ischemic renal[10, 11], myocardial[12-15] and limb tissue injury[16]; support and maintain hematopoiesis[17], support formation of megakaryocyte and pro-platelet[18].

There is an unmet need for an "off-the-shelf" MSC-based therapeutic option, at affordable costs, with better quality control and consistency. This is a requisite for time-sensitive protection against injury such as reperfusion injury in patients with acute MI,.

SUMMARY

According to a 1st aspect of the present invention, we provide a particle secreted by a mesenchymal stem cell and comprising at least one biological property of a mesenchymal stem cell.

The biological property may comprise a biological activity of a mesenchymal stem cell conditioned medium (MSC-CM). The biological activity may comprise cardioprotection. The particle may be capable of reducing infarct size.

Reduction of infarct may be assayed in a mouse or pig model of myocardial ischemia and reperfusion injury.

The particle may be capable of reducing oxidative stress. The reduction of oxidative stress may be assayed in an *in vitro* assay of hydrogen peroxide (H_2O_2)-induced cell death.

The particle comprise a vesicle. The particle may comprise an exosome.

The particle may contain at least 70% of proteins in an mesenchymal stem cell conditioned medium (MSC-CM). The proteins may be selected from the list shown in Table D1 or may be gene products of the genes shown in Table D2.

The particle may comprise a complex of molecular weight >100 kDa. The complex of molecular weight > 100 kDa may comprise proteins of <100 kDa. The particle may comprise a complex of molecular weight > 300 kDa. The complex of molecular weight > 100 kDa may comprise proteins of < 300 kDa.

The particle may comprise a complex of molecular weight > 1000 kDa.The particle may have a size of between 2 nm and 200 nm. The particle may have a size of

between 50ηm and 150 nm. The particle may have a size of between between 50 nm and 100 nm.

The size of the particle may be determined by filtration against a 0.2μM filter and concentration against a membrane with a molecular weight cut-off of 10 kDa. The size of the particle may be determined by electron microscopy.

The particle may comprise a hydrodynamic radius of below 100 nm. It may comprise a hydrodynamic radius of between about 30 nm and about 70 nm. It may be between about 40 nm and about 60 nm, such as between about 45 nm and about 55 nm. The mesenchymal stem cell particle may comprise a hydrodynamic radius of about 50 nm. The hydrodynamic radius may be determined by laser diffraction or dynamic light scattering.

The particle may comprise a lipid selected from the group consisting of: phospholipid, phosphatidyl serine, phosphatidyl inositol, phosphatidyl choline, shingomyelin, ceramides, glycolipid, cerebroside, steroids, cholesterol. The cholesterol-phospholipid ratio may be greater than 0.3-0.4 (mol/mol). The particle may comprise a lipid raft.

The particle may be insoluble in non-ionic detergent, preferably Triton-X100. The particle may be such that proteins of the molecular weights specified substantially remain in the complexes of the molecular weights specified, when the particle is treated with a non-ionic detergent.

The particle may be sensitive to cyclodextrin, preferably 20 mM cyclodextrin. The particle may be such that treatment with cyclodextrin causes substantial dissolution of the complexes specified.

The particle may comprise ribonucleic acid (RNA). The particle may have an absorbance ratio of 1.9 (260:280 nm). The particle may comprise a surface antigen selected from the group consisting of: CD9, CD109 and thy-1.

There is provided, according to a 2nd aspect of the present invention, a method of producing a particle according to any preceding claim, the method comprising isolating the particle from a mesenchymal stem cell conditioned medium (MSC-CM).

The method may comprise separating the particle from other components based on molecular weight, size, shape, composition or biological activity.

The weight may be selected from the weights set out above. The size may be selected from the sizes set out above. The composition may be selected from the compositions set out above. The biological activity may be selected from the biological activities set out above.

We provide, according to a 3rd aspect of the present invention, a method of producing a particle according as described above. The method may comprise obtaining a mesenchymal stem cell conditioned medium (MSC-CM). It may comprise concentrating the mesenchymal stem cell conditioned medium. The mesenchymal stem cell conditioned medium may be concentrated by ultrafiltration over a >1000 kDa membrane. The method may comprise subjecting the concentrated mesenchymal stem cell conditioned medium to size exclusion chromatography. A TSK Guard column SWXL, 6 x 40 mm or a TSK gel G4000 SWXL, 7.8 x 300 mm column may be employed. The method may comprise selecting UV absorbant fractions, for example, at 220 nm, that exhibit dynamic light scattering. The dynamic light scattering may be detected by a quasi-elastic light scattering (QELS) detector. The method may comprise collecting fractions which elute with a retention time of 11-13 minutes, such as 12 minutes.

We provide, according to a 4th aspect of the present invention, a pharmaceutical composition comprising a particle as described together with a pharmaceutically acceptable excipient, diluent or carrier.

As a fourth aspect of the present invention, there is provided such a particle or such a pharmaceutical composition for use in a method of treating a disease.

We provide, according to a 5th aspect of the present invention, use of such a particle for the preparation of a pharmaceutical composition for the treatment of a disease.

The present invention, in a 6th aspect, provides use of such a particle in a method of treatment of a disease in an individual.

The disease may be selected from the group consisting of: cardiac failure, bone marrow disease, skin disease, burns and degenerative diseases such as diabetes, Alzheimer's disease, Parkinson's disease and cancer.

The disease may be selected from the group consisting of: myocardial infarction, a cutaneous wound, a dermatologic disorder, a dermatological lesion, dermatitis, psoriasis, condyloma, verruca, hemangioma, keloid, skin cancer, atopic dermatitis, Behcet disease, chronic granulomatous disease, cutaneous T cell lymphoma, ulceration, a pathological

condition characterised by initial injury inducing inflammation and immune dysregulation leading to chronic tissue remodeling including fibrosis and loss of function, renal ischemic injury, cystic fibrosis, sinusitis and rhinitis or an orthopaedic disease.

The particle may be used to aid wound healing, scar reduction, bone formation, a bone graft or bone marrow transplantation in an individual.

The particle may be used (i) in the regulation of a pathway selected from any one or more of the following: cytoskeletal regulation by Rho GTPase, cell cycle, integrin signalling pathway, Inflammation mediated by chemokine & cytokine signaling pathway, FGF signaling pathway, EGF receptor signaling pathway, angiogenesis, plasminogen activating cascade, blood coagulation, glycolysis, ubiquitin proteasome pathway, de novo purine biosynthesis, TCA cycle, phenylalanine biosynthesis, heme biosynthesis; (ii) in the regulation of processes including any one or more of the following: cell structure and motility, cell structure, cell communication, cell motility, cell adhesion, endocytosis, mitosis, exocytosis, cytokinesis, cell cycle, immunity and defense, cytokine/chemokine mediated immunity, macrophage-mediated immunity, granulocyte-mediated immunity, ligand-mediated signaling, cytokine and chemokine mediated signaling pathway, signal transduction, extracellular matrix protein-mediated signaling, growth factor homeostasis, receptor protein tyrosine kinase signaling pathway, cell adhesion-mediated signaling, cell surface receptor mediated signal transduction, JAK-STAT cascade, antioxidation and free radical removal, homeostasis, stress response, blood clotting, developmental processes, mesoderm development, skeletal development, angiogenesis, muscle development, muscle contraction, protein metabolism and modification, proteolysis, protein folding, protein complex assembly, amino acid activation, intracellular protein traffic, other protein targeting and localization, amino acid metabolism, protein biosynthesis, protein disulfide-isomerase reaction, carbohydrate metabolism, glycolysis, pentose-phosphate shunt, other polysaccharide metabolism, purine metabolism, regulation of phosphate metabolism, vitamin metabolism, amino acid biosynthesis, pre-mRNA processing, translational regulation, mRNA splicing; or (iii) in the supply of functions including any one or more of the following: signaling molecule, chemokine, growth factor, cytokine, interleukin, other cytokine, extracellular matrix, extracellular matrix structural protein, other extracellular matrix, extracellular matrix glycoprotein, protease, metalloprotease, other proteases, protease inhibitor, metalloprotease inhibitor, serine protease inhibitor, oxidoreductase,

dehydrogenase, peroxidase, chaperone, chaperonin, Hsp 70 family chaperone, other chaperones, synthetase, synthase and synthetase, select calcium binding protein, aminoacyl-tRNA synthetase, lyase, isomerase, other isomerase, ATP synthase, hydratase, transaminase, other lyase, other enzyme regulator, select regulatory molecule, actin binding cytoskeletal protein, cytoskeletal protein, non-motor actin binding protein, actin and actin related protein, annexin, tubulin, cell adhesion molecule, actin binding motor protein, intermediate filament, ribonucleoprotein, ribosomal protein, translation factor, other RNA-binding protein, histone, calmodulin related protein, vesicle coat protein.

In a 7th aspect of the present invention, there is provided a delivery system for delivering a particle, comprising a source of particle together with a dispenser operable to deliver the particle to a target.

According to an 8th aspect of the present invention, we provide use of such a delivery system in a method of delivering a particle to a target.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press; Ausubel, F. M. et al. (1995 and periodic supplements; *Current Protocols in Molecular Biology*, ch. 9, 13, and 16, John Wiley & Sons, New York, N.Y.); B. Roe, J. Crabtree, and A. Kahn, 1996, *DNA Isolation and Sequencing: Essential Techniques*, John Wiley & Sons; J. M. Polak and James O'D. McGee, 1990, *Oligonucleotide Synthesis: A Practical Approach*, Irl Press; D. M. J. Lilley and J. E. Dahlberg, 1992, *Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA* Methods in Enzymology, Academic Press; Using Antibodies : A Laboratory Manual : Portable Protocol NO. I by Edward Harlow, David Lane, Ed Harlow (1999, Cold Spring Harbor Laboratory Press, ISBN 0-87969-544-7); Antibodies : A Laboratory Manual by Ed Harlow (Editor), David Lane (Editor) (1988, Cold Spring Harbor Laboratory Press, ISBN 0-87969-314-2), 1855; and Lab Ref: A Handbook of Recipes, Reagents, and Other Reference Tools for Use at the Bench, Edited Jane Roskams and Linda Rodgers, 2002, Cold Spring Harbor Laboratory, ISBN 0-87969-630-3. Each of these general texts is herein incorporated by reference.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Myocardial Infarct Size. Representative pictures of Evans blue (blue) and TTC (red) staining on hearts of pigs treated with non-CM (A), CM (B) or saline (C). Myocardial infarct size quantifications as a percentage of the left ventricle (LV) and the area at risk (AAR) are shown in Figures D and E, respectively. Non-CM, n=9; CM, n=9; saline, n=8.

Figure 2. Local and Global systolic function. Local systolic wall thickening as assessed with echocardiography of the infarct area in pigs treated with Non-CM, CM or saline (A). Global systolic function, echocardiographic fractional area shortening (FAS) is shown in Figure B. Non-CM, n=9; CM, n=9; saline, n=8. * $p < 0.05$ vs. Non-CM and saline.

Figure 3. Oxidative Stress. Viability of CEM cells in either CM or Non-CM and treated with H_2O_2. (* $p<0.05$, A). Conditioned medium protects cells from death induced by H_2O_2. To assess oxidative stress *in vivo*, infarct area sections from pigs treated with Non-CM (B), CM (C), or saline (D) were stained for 8-OHdG, a product of nuclear oxidative stress. Quantification of 8-OHdG positive nuclei was assessed at 200x magnification and is depicted in Figure E. Also *in vivo*, CM reduces oxidative stress. Non-CM, n=9; CM, n=9; saline, n=8.

Figure 4. TGF-β Signaling and Apoptosis. Western blot for phosphorylated SMAD 2 (A, B) and active caspase 3 (C, D) in pigs treated with non-CM, CM or saline. Beta-tubulin was assessed as a loading control, and no differences were found in beta-tubulin expression between the groups (E). Non-CM, n=9; CM, n=9; saline, n=8.

Figure 5. Cardioprotective Properties of CM Fractions. Myocardial infarct size quantification in mice treated with saline, <1000 kD fraction of CM or unfractionated CM. Unfractionated conditioned medium significantly reduces myocardial infarct size compared to saline treated animals. The <1000 kDa fraction, however does not, indicating that the cardioprotective factor(s) is/are larger than 1000 kDa. Saline, n=10; <1000 kD, n=8; unfractionated, n=12. * $p < 0.01$ vs. saline.

Figure 6. Chemically defined medium conditioned by hESC-MSC HuES9.E1 cells for three days was filtered through a 0.22 μfilter. The conditioned medium (CM) was concentrated 25 times by filtering through a membrane with a 10 kD MW cut-off. The concentrated CM was then centrifuged through a membrane with either a 100 kD or a 300

kD MW cut-off resulting in a 4:1 (vol:vol) filtrate to retentate ratio. The unfractionated CM, filtrate and retentate samples were loaded in the volume ratio of 5:4:1

Figure 7. Identity of components in the filtrate after filtration through a membrane with a 100 kD MW cut-off. a) comparison of protein components in filtrate vs protein components in the non-conditioned medium (NCM). Conditioned medium (CM), the filtrate after CM was filtered through membrane with a 100 kD MW cut-off (100 kD filtrate) and NCM was separated on a SDS-PAGE. The gel was stained with silver to visualize protein bands; b) CM was filtered through a 100 kD MW cut-off membrane to produce a retentate to filtrate volume ratio of 4:1. The The retentate, filtrate,and the different protein supplements in the chemically defined medium, namely insulin-transferin-selenoprotein, FGF2, EGF and PDGF AB were separated on a SDS-PAGE The retentate and filtrate was loaded in a volume ratio of 4:1

Figure 8. Relative sizes of biological materials and pore sizes in membranes. Reprint from Spectrum© Laboratory.

Figure 9. Relative AAR in mice after ischemia/reperfusion. Myocardial infarction was induced by left coronary artery (LCA) occlusion by suture ligation for 30 minutes, and reperfusion was initiated by removal of suture. Five minutes before reperfusion, mice were treated with tail vein injection of 20 µl unfractionated MSC-CM (10-220 nm), 20 µl of < 100 or 1,000 kD fraction, 4 µl of >1000 kD retentate or saline. 24 hours later, the hearts were excised. Before excision, the Area At Risk (AAR) was determined by religating the LCA and then perfusing Evans blue through the aorta. AAR was defined as the area not stained by the dye and was expressed as a percentage of the left ventricular wall area.

Figure 10. Effects of conditioned media and fractionated conditioned media on relative infarct size in mice after ischemia/reperfusion. After excision of the heart, infarct size was assessed 24 hours later using TTC and expressed as a percentage of AAR.

Figure 11. Physical entities of between 50-100nm in diameter were observed using electron microscopy.

Figure 12. Size fractionation of CM after treatment with Triton X-100, CM was treated with a final 0.5 or 1.0% (v/v) TritonX-100 for 30 minutes and then fractionate by filtration through a membrane with MW cut-off of 100 kD to generate a filtrate:retentate volume ratio of 4:1.

Figure 13. Proteins found from MS/MS analysis that are common to exosomes from different cell types[44].

Figure 14. Cardioprotective Properties of CM Fractions. Myocardial infarct size quantification in mice treated with saline, HEK293 conditioned medium, unfractionated hESC-CM, CM filtered with MWCO of 100 kDa, 300 kDa, 500 kDa, and 1000 kDa or CM concentrated 50 times against membrane with MWCO of 100 kDa fraction of CM or unfractionated CM.

Figure 15. Immunoprecipitation. CM immunoprecipitated with anti-CD81 or mouse IgG as negative control. The immunoprecipitate and supernatant were analysed by western blot hybridization using antibody against CD9, Alix, Tsp-1, pyruvate kinase and SOD1.

Figure 16. Ultracentrifugation of CM. CM was concentrated five times by filtering through a membrane with MWCO of 500 kDa. The retentate, and the unfiltered CM were ultracentrifuged at 200,000g for 2 hour. The supernatant and the pellet were analysed by western blotting for the presence of CD9. Lane 1-3: different protein concentration of CM. Lane 4 and 5: The pellet (P) and supernatant (S) after ultracentrifugation of unfiltered CM. Lane 6: Retentate after filteration of CM through a membrane with MWCO of 500 kDa. Lane 7-8: The pellet (P) and supernatant (S) after ultracentrifugation of retentate. Lane 9: Filtrate after after filteration of CM through a membrane with MWCO of 500 kDa.

Figure 17. Fractionation on sucrose gradient density. A sucrose density gradient was prepared by layering 14 sucrose solutions of concentrations from 22.8-60% (w/v) in a SW60Ti centrifuge tube with the most concentrated solution at the bottom of the ultracentrifuge tube. The sample was loaded on top of the gradient and ultracentrifuged for 16.5 hours at 200,000X g, 4°C in a SW60Ti rotor (Beckman Coulter Inc.). 13 fractions were collected from top to the bottom of the sucrose gradient. The density of each fraction was calculated by weighing a fixed volume of each fraction. The fractions were then analysed by western blot analysis and probed for pyruvate kinase, CD9, CD81 and SOD1. Protein standard molecular weight markers were fractionated on a similar gradient and the distribution of the markers in the different fractions of the sucrose gradient were denoted at the bottom of the figure. a) CM was fractionated, b) CM was treated with lysis buffer before being fractionated on the sucrose gradient.

Figure 18. Trypsinization of CM. CM was digested with trypsin for 0, 0.5, 2, 10 and 20 mins. The partially digested CM was analysed for the presence of CD9 and SOD1.

Figure 19. Analysis of RNA in the CM. (a, b) RNA was extracted from MSCs and MSC-CM. The purified RNA was denatured and separated on a glyoxal agarose gel and urea-PAGE, respectively. (c) Equal volume of CM was treated with PBS, cyclodextrin, lysis buffer or phospholipase A2. The untreated and treated CM were extracted for RNA. The RNA was separated on urea-PAGE. (d) RNA from CM was treated with RNAse III and the treated RNA was separated in parallel with untreated RNA on a urea-PAGE. RNA in the gels was visualized by staining with ethidium bromide.

Figure 20. Density of RNA in the CM. After fractionation of CM, CM pretreated with lysis buffer and RNA MW standards on a sucrose density gradient equilibrium centrifugation as described in figure 4, each fraction was extracted for RNA. a) the relative RNA yield for each fraction in each sample was plotted against fraction number. The relative concentration was normalized to the highest RNA concentration in each gradient which was arbitrary set as 100%. b) The RNA extracted from each fraction was separated on a urea-PAGE.

Figure 21. RNA was not in CD81+ exosomes. CD81 immunoprecipitation which also precipitated CD9 was performed as described in fig 2. The immunoprecipitate and the supernatant were extracted for RNA and the extracts were separated on a urea-PAGE and visualized by ethidium bromide staining.

Figure 22. Microarray analysis of miRNA in the CM. RNA samples from MSC and the CM were hybridized to microarray chips containing probes for miRNA transcripts listed in Sanger miRBase Release 10.1. The hybridization was performed using two biological replicates of each RNA samples. a) 149 miRNA were detected in MSC and 63 were detected in the CM. Of these, 47 were expressed at similar levels in both MSCs and the CM. 16 were expressed at a detectable level in CM but not in MSC s while 47 were detected in MSCs but not in the CM. b) Relative expression level of guide miRNA to its anti-guide miRNA based on hybridization signal on the microarray chip.

Figure 23. HPLC fractionation of CM and NCM. CM and NCM were fractionated on a HPLC using BioSep S4000, 7.8 mm x 30 cm column. The components in CM or NCM were eluted with 20 mM phosphate buffer with 150 mM of NaCl at pH 7.2. The elution mode was isocratic and the run time was 40 minutes. The eluent was monitored

with an UV-visible detector set at 220 nm. The % area under each peak was integrated from the UV-visible detector.

DETAILED DESCRIPTION

The present invention is based on the demonstration that human ESC-derived mesenchymal stem cells (MSCs) mediate cardioprotective effects through secreted large complexes of ~50-100 nm in diameter. Such complexes or particles may therefore be used for therapeutic means, including for cardioprotection, in place of the cells themselves.

The Examples describe proteomic analysis of these complexes, revealing the presence of exosome-associated proteins e.g. CD81, CD9 and Alix that also co-immunopreciptate, and of membrane and cytosolic proteins that exhibited detergent-sensitive proteolysis consistent with-membrane-bound and membrane-encapsulated proteins respectively.

The Examples further demonstrate other properties of these particles or complexes. We show that proteins of such particles or complexes have MW-independent sedimentation densities of 1.016- 1.215 g/ml that revert to MW-dependent densities upon treatment with a membrane lysis buffer. The secretion also contains RNAs (<300 nts) in cholesterol-rich lipid vesicles. HPLC fractionation and dynamic light scattering studies further indicate that the only detectable particles in the secretion within hydrodynamic radius (rh) range of 1-1000 nm had a rh of 45-55 nm.

These observations together with the presence of membrane lipids e.g. cholesterol sphingomyelin and phosphatidylcholine demonstrate that the cardioprotective complexes in the secretion are exosomes or secreted lipid vesicles.

These mesenchymal stem cell particles, complexes or exosomes may be used for a variety of purposes, such as treatment or prevention for cardiac or heart diseases such as ischaemia, cardiac inflammation or heart failure. They may also be used for repair following perfusion injury.

PARTICLE

We describe a particle which is derivable from a mesenchymal stem cell (MSC).

The particle may be derivable from the MSC by any of several means, for example by secretion, budding or dispersal from the MSC. For example, the particle may be

produced, exuded, emitted or shed from the MSC. Where the MSC is in cell culture, the particle may be secreted into the cell culture medium.

The particle may in particular comprise a vesicle. The particle may comprise an exosome. The particles described here may comprise any one or more of the properties of the exosomes described herein.

The particle may comprise vesicles or a flattened sphere limited by a lipid bilayer. The particles may comprise diameters of 40-100 nm. The particles may be formed by inward budding of the endosomal membrane. The particles may have a density of ~1.13 – 1.19 g/ml and may float on sucrose gradients. The particles may be enriched in cholesterol and sphingomyelin, and lipid raft markers such as GM1, GM3, flotillin and the src protein kinase Lyn. The particles may comprise one or more proteins present in mesenchymal stem cells or mesenchymal stem cell conditioned medium (MSC-CM), such as a protein characteristic or specific to the MSC or MSC-CM. They may comprise RNA, for example miRNA.

We provide a particle which comprises one or more genes or gene products found in MSCs or medium which is conditioned by culture of MSCs. The particle may comprise molecules secreted by the MSC. Such a particle, and combinations of any of the molecules comprised therein, including in particular proteins or polypeptides, may be used to supplement the activity of, or in place of, the MSCs or medium conditioned by the MSCs for the purpose of for example treating or preventing a disease.

The particle may comprise a cytosolic protein found in cytoskeleton e.g. tubulin, actin and actin-binding proteins, intracellular membrane fusions and transport e.g. annexins and rab proteins, signal transduction proteins e.g. protein kinases, 14-3-3 and heterotrimeric G proteins, metabolic enzymes e.g. peroxidases, pyruvate and lipid kinases, and enolase-1 and the family of tetraspanins e.g. CD9, CD63, CD81 and CD82. In particular, the particle may comprise one or more tetraspanins. The particles may comprise mRNA and/or microRNA.

The term “particle” as used in this document may be taken to mean a discrete entity. The particle may be something that is isolatable from a mesenchymal stem cell (MSC) or mesenchymal stem cell conditioned medium (MSC-CM). The particle may be responsible for at least an activity of the MSC or MSC-CM. The particle may be responsible for, and carry out, substantially most or all of the functions of the MSC or

MSC-CM. For example, the particle may be a substitute (or biological substitute) for the MSC or MSC-CM.

The particle may be used for any of the therapeutic purposes that the MSC or MSC-CM may be put to use.

The particle preferably has at least one property of a mesenchymal stem cell. The particle may have a biological property, such as a biological activity. The particle may have any of the biological activities of an MSC. The particle may for example have a therapeutic or restorative activity of an MSC.

The Examples show that media conditioned by MSCs (such as mesenchymal stem cell conditioned media or MSC-CM, as described below) comprise biological activities of MSC and are capable of substituting for the MSCs themselves. The biological property or biological activity of an MSC may therefore correspond to a biological property or activity of an mesenchymal stem cell conditioned medium. Accordingly, the particle may comprise one or more biological properties or activities of a mesenchymal stem cell conditioned medium (MSC-CM).

Mesenchymal Stem Cell Conditioned Medium (MSC-CM)

The conditioned cell culture medium such as a Mesenchymal Stem Cell Conditioned Medium (MSC-CM) may be obtained by culturing a mesenchymal stem cell (MSC), a descendent thereof or a cell line derived therefrom in a cell culture medium; and isolating the cell culture medium. The mesenchymal stem cell may be produced by a process comprising obtaining a cell by dispersing a embryonic stem (ES) cell colony. The cell, or a descendent thereof, may be propagated in the absence of co-culture in a serum free medium comprising FGF2.

Mesenchymal Stem Cell Particle

The particle may be produced or isolated in a number of ways. Such a method may comprise isolating the particle from a mesenchymal stem cell (MSC). Such a method may comprise isolating the particle from an mesenchymal stem cell conditioned medium (MSC-CM).

The particle may be isolated for example by being separated from non-associated components based on any property of the particle. For example, the particle may be isolated based on molecular weight, size, shape, composition or biological activity.

The conditioned medium may be filtered or concentrated or both during, prior to or subsequent to separation. For example, it may be filtered through a membrane, for example one with a size or molecular weight cut-off. It may be subject to tangential force filtration or ultrafiltration.

For example, filtration with a membrane of a suitable molecular weight or size cutoff, as described in the Assays for Molecular Weight elsewhere in this document, may be used.

The conditioned medium, optionally filtered or concentrated or both, may be subject to further separation means, such as column chromatography. For example, high performance liquid chromatography (HPLC) with various columns may be used. The columns may be size exclusion columns or binding columns.

One or more properties or biological activities of the particle may be used to track its activity during fractionation of the mesenchymal stem cell conditioned medium (MSC-CM). As an example, light scattering, refractive index, dynamic light scattering or UV-visible detectors may be used to follow the particles. For example, a therapeutic activity such as cardioprotective activity may be used to track the activity during fractionation.

The following paragraphs provide a specific example of how a mesenchymal stem cell particle such as an exosome may be obtained.

A mesenchymal stem cell particle may be produced by culturing mesenchymal stem cells in a medium to condition it. The mesenchymal stem cells may comprise HuES9.E1 cells. The medium may comprise DMEM. The DMEM may be such that it does not comprise phenol red. The medium may be supplemented with insulin, transferrin, or selenoprotein (ITS), or any combination thereof. It may comprise FGF2. It may comprise PDGF AB. The concentration of FGF2 may be about 5 ng/ml FGF2. The concentration of PDGF AB may be about 5 ng/ml. The medium may comprise glutamine-penicillin-streptomycin or b-mercaptoethanol, or any combination thereof.

The cells may be cultured for about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 days or more, for example 3 days. The conditioned medium may be obtained by separating the cells from the medium. The conditioned medium may be centrifuged, for example at 500 g. it may be concentrated by filtration through a membrane. The membrane may comprise a > 1000 kDa membrame. The conditioned medium may be concentrated about 50 times or more.

The conditioned medium may be subject to liquid chromatography such as HPLC. The conditioned medium may be separated by size exclusion. Any size exclusion matrix such as Sepharose may be used. As an example, a TSK Guard column SWXL, 6 x 40 mm or a TSK gel G4000 SWXL, 7.8 x 300 mm may be employed. The eluent buffer may comprise any physiological medium such as saline. It may comprise 20 mM phosphate buffer with 150 mM of NaCl at pH 7.2. The chromatography system may be equilibrated at a flow rate of 0.5 ml/min. The elution mode may be isocratic. UV absorbance at 220 nm may be used to track the progress of elution. Fractions may be examined for dynamic light scattering (DLS) using a quasi-elastic light scattering (QELS) detector.

Fractions which are found to exhibit dynamic light scattering may be retained. For example, a fraction which is produced by the general method as described above, and which elutes with a retention time of 11-13 minutes, such as 12 minutes, is found to exhibit dynamic light scattering. The r_h of particles in this peak is about 45-55 nm. Such fractions comprise mesenchymal stem cell particles such as exosomes.

PARTICLE PROPERTIES

The property of a mesenchymal stem cell may comprise a property of a medium conditioned by a mesenchymal stem cell (MSC-CM). Methods of producing such a mesenchymal stem cell conditioned medium are described elsewhere in this document and are illustrated in for example Example 1 below.

The property may comprise a biological property such as a biological activity. Examples of biological activities include cardioprotection, reduction of oxidative stress and reduction of infarct size.

CARDIOPROTECTION

The particle may have a property of mesenchymal stem cells and/or mesenchymal stem cell conditioned medium (MSC-CM) comprising cardioprotection The cardioprotection may comprise restoration or maintenance of cardiac function during ischemia and/or reperfusion.

Assay for Cardioprotection

Cardioprotection may for example be assayed using any one or more of the methods described in Examples 5, 10, 14 and 20.

OXIDATIVE STRESS

The particle may have a property of mesenchymal stem cells and/or mesenchymal stem cell conditioned medium (MSC-CM) comprising the ability to reduce oxidative stress (or cytoprotection).

Assay for Oxidative Stress

The reduction of oxidative stress may for example be assayed using an *in vitro* assay of hydrogen peroxide (H_2O_2)-induced cell death. In summary, hydrogen peroxide (H_2O_2)-mediated oxidative stress is induced in human leukemic CEM cells and cell viability is monitored by Trypan blue-exclusion. Human leukemic CEM cells are incubated with particle, conditioned medium or mesenchymal stem cell (with saline as a control) and treated with 50 μM H_2O_2 to induce oxidative stress. Cell viability is assessed using Trypan Blue exclusion at 12, 24, 36 and 48 hours after H_2O_2 treatment.

The reduction of oxidative stress may further be assayed using an *in vivo* assay of DNA oxidation. *In vivo* oxidative stress may also be assayed as follows. Pigs are treated with the particle, conditioned medium or mesenchymal stem cell (with saline as a control). Tissue sections of pig heart are obtained. Nuclear oxidative stress in tissue sections of treated and untreated pigs is quantified by 8-OHdG immunostaining for oxidized DNA. The tissue sections are assayed for intense nuclear staining indicative of DNA oxidation and oxidative stress.

INFARCT SIZE

The particle may have a property of mesenchymal stem cells and/or mesenchymal stem cell conditioned medium (MSC-CM) comprising the ability to reduce infarct size.

Assay for Infarct Size

Infarct size may for example be assayed using any one or more of the methods described in Examples 6 and 13.

PARTICLE MOLECULAR WEIGHT

The particle may have a molecular weight of greater than 100 kDa. It may have a molecular weight of greater than 500 kDa. For example, it may have a molecular weight of greater than 1000 kDa.

The molecular weight may be determined by various means. In principle, the molecular weight may be determined by size fractionation and filtration through a

membrane with the relevant molecular weight cut-off. The particle size may then be determined by tracking segregation of component proteins with SDS-PAGE or by a biological assay.

Assay of Molecular Weight by SDS-PAGE

The particle may have a molecular weight of greater than 100 kDa. For example, the particle may be such that most proteins of the particle with less than 100 kDa molecular weight segregate into the greater than 100 kDa molecular weight retentate fraction, when subject to filtration. Similarly, when subjected to filtration with a membrane with a 500 kDa cut off, most proteins of the particle with less than 500 kDa molecular weight may segregate into the greater than 500 kDa molecular weight retentate fraction. This indicates that the particle may have a molecular weight of more than 500 kDa.

Assay of Molecular Weight by Biological Activity

The particle may have a molecular weight of more than 1000 kDa. For example, the particle may be such that when subject to filtration with a membrane with a molecular weight cutoff of 1000 kDa, the relevant biological activity substantially or predominantly remains in the retentate fraction. Alternatively or in addition, biological activity may be absent in the filtrate fraction. The biological activity may comprise any of the biological activities of the particle described elsewhere in this document.

Assay of Molecular Weight by Infarct Size

For example, the biological activity may comprise reduction of infarct size, as assayed in any suitable model of myocardia ischemia and reperfusion injury. For example, the biological activity may be assayed in a mouse or pig model, such as described in the Examples.

In summary, myocardial ischemia is induced by 30 minutes left coronary artery (LCA) occlusion by suture ligation and reperfusion is initiated by removal of suture. Mice are treated with liquid containing the particles (such as unfractionated MSC-CM), filtrate (such as < 100 or 1,000 kD fraction), retentate (such as >1000 kD retentate) or saline intravenously via the tail vein, 5 minutes before reperfusion. 24 hours later, the hearts are excised. Before excision, the Area At Risk (AAR) is determined by religating the LCA and then perfusing Evans blue through the aorta.

AAR is defined as the area not stained by the dye and is expressed as a percentage of the left ventricular wall area. Infarct size is assessed 24 hours later using Evans blue and TTC. Where the relative infarct size is significantly reduced in animals treated with mesenchymal stem cell conditioned medium (MSC-CM) and the retentate (such as a >1000 kD) fraction when compared to saline, this indicates that the particle has a molecular weight which is higher than the relevant cutoff of the membrane (e.g., greater than 1000 kDa).

PARTICLE SIZE

The particle may have a size of greater than 2 nm. The particle may have a size of greater than 5 nm, 10 nm, 20 nm, 30 nm, 40 nm or 50 nm. The particle may have a size of greater than 100 nm, such as greater than 150 nm. The particle may have a size of substantially 200 nm or greater.

The particle or particles may have a range of sizes, such as between 2 nm to 20 nm, 2 nm to 50 nm, 2 nm to 100 nm, 2 nm to 150 nm or 2 nm to 200 nm. The particle or particles may have a size between 20 nm to 50 nm, 20 nm to 100 nm, 20 nm to 150 nm or 20 nm to 200 nm. The particle or particles may have a size between 50 nm to 100ηm, 50ηm to 150 nm or 50 nm to 200 nm. The particle or particles may have a size between 100 nm to 150 nm or 100 nm to 200 nm. The particle or particles may have a size between 150 nm to 200 nm.

The size may be determined by various means. In principle, the size may be determined by size fractionation and filtration through a membrane with the relevant size cut-off. The particle size may then be determined by tracking segregation of component proteins with SDS-PAGE or by a biological assay.

The size may also be determined by electron microscopy, as described in Example 21.

The size may comprise a hydrodynamic radius. The hydrodynamic radius of the particle may be below 100 nm. It may be between about 30 nm and about 70 nm. The hydrodynamic radius may be between about 40 nm and about 60 nm, such as between about 45 nm and about 55 nm. The hydrodynamic radius may be about 50 nm.

The hydrodynamic radius of the particle may be determined by any suitable means, for example, laser diffraction or dynamic light scattering. An example of a dynamic light scattering method to determine hydrodynamic radius is set out in Example 33 below.

COMPOSITION

The particle may comprise one or more proteins secreted by a mesenchymal stem cell. The particle may comprise one or more proteins present in mesenchymal stem cell conditioned medium (MSC-CM).

For example, the particle may comprise 10% or more, 20% or more, 30% or more, 40% or more, 50% or more, 60% or more or 70% or more of these proteins. The particle may comprise substantially about 75% of these proteins. The proteins may be defined by reference to a list of proteins or gene products of a list of genes.

Proteins

The proteins may be selected from those set out in Table D1 below. Table D1 comprises the following proteins numbered 1 to 250, as well as the "proteins with unidentified functions", in the paragraphs below:

1. IPI00021428 Actin, alpha skeletal muscle; 2. IPI00414057 Actin alpha 1 skeletal muscle protein; 3. IPI00008603 Actin, aortic smooth muscle; 4. IPI00021439 Actin, cytoplasmic 1; 5. IPI00023006 Actin, alpha cardiac; 6. IPI00021440 Actin, cytoplasmic 2; 7. IPI00025416 Actin, gamma-enteric smooth muscle; 8. IPI00479925 agrin; 9. IPI00015102 CD166 antigen precursor; 10. IPI00007423 Acidic leucine-rich nuclear phosphoprotein 32 family member B; 11. IPI00413331 36 kDa protein; 12. IPI00412577 34 kDa protein; 13. IPI00413506 33 kDa protein; 14. IPI00418169 Hypothetical protein DKFZp686P03159; 15. IPI00003815 Rho GDP-dissociation inhibitor 1; 16. IPI00004656 Beta-2-microglobulin precursor; 17. IPI00218042 Splice Isoform BMP1-5 of Bone morphogenetic protein 1 precursor; 18. IPI00009054 Splice Isoform BMP1-3 of Bone morphogenetic protein 1 precursor; 19. IPI00014021 Splice Isoform BMP1-1 of Bone morphogenetic protein 1 precursor; 20. IPI00218040 Splice Isoform BMP1-4 of Bone morphogenetic protein 1 precursor; 21. IPI00006980 Protein C14orf166; 22. IPI00296165 Complement C1r subcomponent precursor; 23. IPI00152540 OTTHUMP00000016748; 24. IPI00305064 Splice Isoform CD44 of CD44 antigen precursor; 25. IPI00297160 Hypothetical protein DKFZp451K1918; 26. IPI00293539 Splice Isoform 2 of Cadherin-11 precursor; 27. IPI00304227 Splice Isoform 1 of Cadherin-11 precursor; 28. IPI00386476 Cadherin 11, type 2, isoform 1 preproprotein; 29. IPI00024046 Cadherin-13 precursor;

30. IPI00290085 Neural-cadherin precursor; 31. IPI00029739 Splice Isoform 1 of Complement factor H precursor; 32. IPI00012011 Cofilin, non-muscle isoform; 33. IPI00007257 calsyntenin 1 isoform 2; 34. IPI00218539 Splice Isoform B of Collagen alpha-1(XI) chain precursor; 35. IPI00477350 Collagen, type XI, alpha 1; 36. IPI00329573 Splice Isoform Long of Collagen alpha-1(XII) chain precursor; 37. IPI00221384 Splice Isoform Short of Collagen alpha-1(XII) chain precursor; 38. IPI00400935 Collagen alpha-1(XVI) chain precursor; 39. IPI00297646 Collagen alpha-1(I) chain precursor; 40. IPI00164755 Prepro-alpha2(I) collagen precursor; 41. IPI00304962 Collagen alpha-2(I) chain precursor; 42. IPI00021033 Collagen alpha-1(III) chain precursor; 43. IPI00167087 COL3A1 protein; 44. IPI00021034 Collagen alpha-1(IV) chain precursor; 45. IPI00479324 alpha 2 type IV collagen preproprotein; 46. IPI00306322 Collagen alpha-2(IV) chain precursor; 47. IPI00303313 Collagen alpha-1(V) chain precursor; 48. IPI00477611 184 kDa protein; 49. IPI00293881 COL5A2 protein; 50. IPI00018279 Collagen alpha-3(V) chain precursor; 51. IPI00291136 Collagen alpha-1(VI) chain precursor; 52. IPI00304840 Splice Isoform 2C2 of Collagen alpha-2(VI) chain precursor; 53. IPI00220613 Splice Isoform 2C2A of Collagen alpha-2(VI) chain precursor; 54. IPI00022200 alpha 3 type VI collagen isoform 1 precursor; 55. IPI00072918 alpha 3 type VI collagen isoform 4 precursor; 56. IPI00220701 Splice Isoform 2 of Collagen alpha-3(VI) chain precursor; 57. IPI00072917 alpha 3 type VI collagen isoform 3 precursor; 58. IPI00021828 Cystatin B; 59. IPI00007778 Di-N-acetylchitobiase precursor; 60. IPI00295741 Cathepsin B precursor;

61. IPI00299219 Protein CYR61 precursor; 62. IPI00514900 42 kDa protein; 63. IPI00333770 Similar to Dedicator of cytokinesis protein 10; 64. IPI00478332 Similar to Dedicator of cytokinesis protein 9; 65. IPI00000875 Elongation factor 1-gamma; 66. IPI00465248 Alpha-enolase; 67. IPI00013769 Alpha-enolase, lung specific; 68. IPI00216171 Gamma-enolase; 69. IPI00218803 Splice Isoform B of Fibulin-1 precursor; 70. IPI00296537 Splice Isoform C of Fibulin-1 precursor; 71. IPI00328113 Fibrillin-1 precursor; 72. IPI00019439 fibrillin 2 precursor; 73. IPI00385645 Splice Isoform 2 of Fibroblast growth factor 17 precursor; 74. IPI00216602 Splice Isoform 5 of Fibroblast growth factor receptor 2 precursor; 75. IPI00216604 Splice Isoform 8 of Fibroblast growth factor receptor 2 precursor; 76. IPI00034099 Hypothetical protein FLJ21918; 77. IPI00333541 Filamin-A; 78. IPI00302592 Filamin A, alpha; 79. IPI00339227

Hypothetical protein DKFZp686O1166; 80. IPI00414283 Fibronectin precursor (FN) (Cold-insoluble globulin) (CIG). Splice isoform 3; 81. IPI00339225 Splice Isoform 5 of Fibronectin precursor; 82. IPI00339319 Splice Isoform 11 of Fibronectin precursor; 83. IPI00556632 Splice Isoform 12 of Fibronectin precursor; 84. IPI00411462 Hypothetical protein DKFZp686B18150; 85. IPI00029723 Follistatin-related protein 1 precursor; 86. IPI00005401 Polypeptide N-acetylgalactosaminyltransferase 5; 87. IPI00219025 Glutaredoxin-1; 88. IPI00171411 Golgi phosphoprotein 2; 89. IPI00026314 Gelsolin precursor;

90. IPI00219757 Glutathione S-transferase P; 91. IPI00027569 Heterogeneous nuclear ribonucleoprotein C-like 1; 92. IPI00003881 HNRPF protein; 93. IPI00442294 Splice Isoform 1 of Neurotrimin precursor; 94. IPI00003865 Splice Isoform 1 of Heat shock cognate 71 kDa protein; 95. IPI00037070 Splice Isoform 2 of Heat shock cognate 71 kDa protein; 96. IPI00220362 10 kDa heat shock protein, mitochondrial; 97. IPI00024284 Basement membrane-specific heparan sulfate proteoglycan core protein precursor; 98. IPI00297284 Insulin-like growth factor binding protein 2 precursor; 99. IPI00297284 Insulin-like growth factor binding protein 2 precursor; 100. IPI00029236 Insulin-like growth factor binding protein 5 precursor; 101. IPI00029236 Insulin-like growth factor binding protein 5 precursor; 102. IPI00029235 Insulin-like growth factor binding protein 6 precursor; 103. IPI00029235 Insulin-like growth factor binding protein 6 precursor; 104. IPI00016915 Insulin-like growth factor binding protein 7 precursor; 105. IPI00016915 Insulin-like growth factor binding protein 7 precursor; 106. IPI00328163 K-ALPHA-1 protein; 107. IPI00021396 Vascular endothelial growth factor receptor 2 precursor; 108. IPI00298281 Laminin gamma-1 chain precursor; 109. IPI00219219 Galectin-1; 110. IPI00023673 Galectin-3 binding protein precursor; 111. IPI00021405 Splice Isoform A of Lamin-A/C; 112. IPI00216953 Splice Isoform ADelta10 of Lamin-A/C; 113. IPI00180173 PREDICTED: similar to tropomyosin 4; 114. IPI00401614 PREDICTED: similar to FKSG30; 115. IPI00374397 PREDICTED: similar to tropomyosin 4; 116. IPI00374732 PREDICTED: similar to PPIA protein; 117. IPI00402104 PREDICTED: similar to peptidylprolyl isomerase A isoform 1; cyclophilin A; peptidyl-pro; 118. IPI00455415 PREDICTED: similar to Heterogeneous nuclear ribonucleoprotein C-like dJ845O24.4; 119. IPI00454722 PREDICTED: similar to

Phosphatidylethanolamine-binding protein; 120. IPI00454852 PREDICTED: similar to Teratocarcinoma-derived growth factor 1;

121. IPI00002802 Protein-lysine 6-oxidase precursor; 122. IPI00410152 latent transforming growth factor beta binding protein 1 isoform LTBP-1L; 123. IPI00220249 Latent transforming growth factor beta-binding protein, isoform 1L precursor; 124. IPI00220249 Latent transforming growth factor beta-binding protein, isoform 1L precursor "; 125. IPI00410152 latent transforming growth factor beta binding protein 1 isoform LTBP-1L; 126. IPI00020986 Lumican precursor; 127. IPI00291006 Malate dehydrogenase, mitochondrial precursor; 128. IPI00005707 Macrophage mannose receptor 2 precursor; 129. IPI00020501 Myosin-11; 130. IPI00019502 Myosin-9; 131. IPI00604620 Nucleolin; 132. IPI00220740 Splice Isoform 2 of Nucleophosmin; 133. IPI00219446 Phosphatidylethanolamine-binding protein; 134. IPI00299738 Procollagen C-endopeptidase enhancer 1 precursor; 135. IPI00015902 Beta platelet-derived growth factor receptor precursor; 136. IPI00216691 Profilin-1; 137. IPI00169383 Phosphoglycerate kinase 1; 138. IPI00219568 Phosphoglycerate kinase, testis specific; 139. IPI00296180 Urokinase-type plasminogen activator precursor; 140. IPI00215943 Splice Isoform 3 of Plectin 1; 141. IPI00215942 Splice Isoform 2 of Plectin 1; 142. IPI00014898 Splice Isoform 1 of Plectin 1; 143. IPI00398777 plectin 1 isoform 8; 144. IPI00398776 plectin 1 isoform 7; 145. IPI00186711 plectin 1 isoform 6; 146. IPI00420096 plectin 1 isoform 3; 147. IPI00398779 plectin 1 isoform 11; 148. IPI00398778 plectin 1 isoform 10; 149. IPI00398002 plectin 1 isoform 1; 150. IPI00419585 Peptidyl-prolyl cis-trans isomerase A;

151. IPI00472718 peptidylprolyl isomerase A isoform 2; 152. IPI00000874 Peroxiredoxin-1; 153. IPI00024915 Peroxiredoxin-5, mitochondrial precursor; 154. IPI00375306 peroxiredoxin 5 precursor, isoform b; 155. IPI00012503 Splice Isoform Sap-mu-0 of Proactivator polypeptide precursor; 156. IPI00374179 proteasome activator subunit 1 isoform 2; 157. IPI00030154 Proteasome activator complex subunit 1; 158. IPI00168812 PTK7 protein tyrosine kinase 7 isoform d precursor; 159. IPI00419941 PTK7 protein tyrosine kinase 7 isoform a precursor; 160. IPI00003590 Quiescin Q6, isoform a; 161. IPI00015916 Bone-derived growth factor (Fragment); 162. IPI00015916 Bone-derived growth factor; 163. IPI00298289 Splice Isoform 2 of Reticulon-4; 164. IPI00021766 Splice Isoform 1 of Reticulon-4; 165. IPI00013895 Calgizzarin; 166.

IPI00010402 Hypothetical protein; 167. IPI00218733 Superoxide dismutase; 168. IPI00014572 SPARC precursor; 169. IPI00005614 Splice Isoform Long of Spectrin beta chain, brain 1; 170. IPI00008780 Stanniocalcin-2 precursor; 171. IPI00301288 SEL-OB protein; 172. IPI00216138 Transgelin; 173. IPI00018219 Transforming growth factor-beta-induced protein ig-h3 precursor; 174. IPI00304865 transforming growth factor, beta receptor III "; 175. IPI00296099 Thrombospondin-1 precursor; 176. IPI00032292 Metalloproteinase inhibitor 1 precursor; 177. IPI00027166 Metalloproteinase inhibitor 2 precursor; 178. IPI00220828 Thymosin beta-4; 179. IPI00180240 thymosin-like 3;

180. IPI00299633 OTTHUMP00000031270 (Fragment); 181. IPI00465028 Triosephosphate isomerase 1 variant (Fragment); 182. IPI00451401 Splice Isoform 2 of Triosephosphate isomerase; 183. IPI00010779 Tropomyosin 4; 184. IPI00216975 Splice Isoform 2 of Tropomyosin alpha-4 chain; 185. IPI00180675 Tubulin alpha-3 chain; 186. IPI00218343 Tubulin alpha-6 chain; 187. IPI00216298 Thioredoxin; 188. IPI00472175 CDNA FLJ46672 fis, clone TRACH3009008, highly similar to Thioredoxin reductase; 189. IPI00450472 Ubiquitin-conjugating enzyme E2I; 190. IPI00018352 Ubiquitin carboxyl-terminal hydrolase isozyme L1; 191. IPI00010207 Ubiquitin-fold modifier 1 precursor; 192. IPI00260630 URB; 193. IPI00021263 14-3-3 protein zeta/delta; 194. IPI00642991 Hypothetical protein DKFZp686F10164; 195. IPI00470919 Hypothetical protein DKFZp686K08164; 196. IPI00719088 collagen, type VI, alpha 1 precursor; 197. IPI00654685 Similar to SPARC precursor; 198. IPI00641961 Collagen, type XII, alpha 1; 199. IPI00645849 Extracellular matrix protein 1; 200. IPI00554786 Thioredoxin reductase 1; 201. IPI00645018 Plasminogen activator, urokinase; 202. IPI00552339 Tissue inhibitor of metalloproteinase 1; 203. IPI00642997 Actin, cytoplasmic 2; 204. IPI00719778 Similar to Annexin A2; 205. IPI00647915 Transgelin 2; 206. IPI00552815 Collagen, type V, alpha 1; 207. IPI00552981 CDNA PSEC0266 fis, clone NT2RP3003649, highly similar to Homo sapiens fibulin-1D mRNA; 208. IPI00180776 29 kDa protein; 209. IPI00552416 Filamin A, alpha;

210. IPI00640698 Actin, gamma-enteric smooth muscle; 211. IPI00514530 Actin, alpha 1, skeletal muscle; 212. IPI00556442 Insulin-like growth factor binding protein 2 variant (Fragment); 213. IPI00513782 Gelsolin; 214. IPI00478731 29 kDa protein; 215. IPI00396479 24 kDa protein; 216. IPI00334627 39 kDa protein; 217. IPI00555762 PTK7 protein tyrosine kinase 7 isoform a variant (Fragment); 218. IPI00658202 97 kDa protein;

219. IPI00006273 CYR61 protein; 220. IPI00719405 TMSL6 protein; 221. IPI00658096 Thymosin beta-4; 222. IPI00376163 5 kDa protein; 223. IPI00556217 Fibrillin 1 variant (Fragment); 224. IPI00514817 Similar to Lamin A/C; 225. IPI00644087 Progerin; 226. IPI00655812 Rhabdomyosarcoma antigen MU-RMS-40.12; 227. IPI00604517 Similar to Nucleolin; 228. IPI00444262 CDNA FLJ45706 fis, clone FEBRA2028457, highly similar to Nucleolin; 229. IPI00412473 Protein; 230. IPI00414489 Protein; 231. IPI00411463 Protein; 232. IPI00556415 Transgelin variant (Fragment); 233. IPI00718825 Calmodulin; 234. IPI00478156 17 kDa protein; 235. IPI00386621 CALM3 protein; 236. IPI00647001 Acidic; 237. IPI00642650 Similar to Stanniocalcin 2 precursor; 238. IPI00641471 Collagen-like protein; 239. IPI00514669 SH3 domain binding glutamic acid-rich protein like 3; 240. IPI00719422 Triosephosphate isomerase (Fragment); 241. IPI00003734 Putative S100 calcium-binding protein H_NH0456N16.1; 242. IPI00029574 11 kDa protein; 243. IPI00641047 Gelsolin; 244. IPI00647556 Gelsolin; 245. IPI00654821 hypothetical protein LOC54845 isoform 1; 246. IPI00647572 Dickkopf related protein-3 precursor; 247. IPI00639879 Similar to Cytokinesis protein sepA; 248. IPI00657746 Similar to Dedicator of cytokinesis protein 8; 249. IPI00555993 Vascular endothelial growth factor receptor 3 variant; 250. IPI00552545 Dedicator of cytokinesis protein 8.

Proteins with unidentified functions: IPI00642991 Hypothetical protein DKFZp686F10164; IPI00470919 Hypothetical protein DKFZp686K08164; IPI00654685 Similar to SPARC precursor; IPI00719778 Similar to Annexin A2; IPI00552981 CDNA PSEC0266 fis, clone NT2RP3003649, highly similar to Homo sapiens fibulin-1D mRNA; IPI00180776 29 kDa protein; IPI00478731 29 kDa protein; IPI00396479 24 kDa protein; IPI00334627 39 kDa protein; IPI00658202 97 kDa protein; IPI00376163 5 kDa protein; IPI00514817 Similar to Lamin A/C; IPI00644087 Progerin; IPI00655812 Rhabdomyosarcoma antigen MU-RMS-40.12; IPI00604517 Similar to Nucleolin; IPI00444262 CDNA FLJ45706 fis, clone FEBRA2028457, highly similar to Nucleolin; IPI00412473 Protein; IPI00414489 Protein; IPI00411463 Protein; IPI00478156 17 kDa protein; IPI00386621 CALM3 protein; IPI00647001 Acidic; IPI00642650 Similar to Stanniocalcin 2 precursor; IPI00641471 Collagen-like protein; IPI00514669 SH3 domain binding glutamic acid-rich protein like 3; IPI00003734 Putative S100 calcium-binding protein H_NH0456N16.1; IPI00029574 11 kDa protein; IPI00654821 hypothetical protein LOC54845 isoform 1; IPI00647572 Dickkopf related protein-3 precursor; IPI00639879

Similar to Cytokinesis protein sepA ; IPI00657746 Similar to Dedicator of cytokinesis protein 8 ; IPI00555993 Vascular endothelial growth factor receptor 3 variant.

Gene Products

The proteins may be selected from the gene products of the genes set out in Table D2 below. Table D2 comprises the following genes in the paragraphs below:

ACTA1; COL5A2; HSPA8; PSAP; ACTA2; COL5A3; HSPE1; PSME1; ACTB; COL6A1; HSPG2; PTK7; ACTC; COL6A2; IGFBP2; QSCN6; ACTG1; COL6A3; IGFBP5; RTN4; ACTG2; CSTB; IGFBP6; S100A11; AGRN; CTBS; IGFBP7; SH3BGRL3; ALCAM; CTSB; K-ALPHA-1; SOD1; ANP32B; CYR61; KDR; SPARC; ANXA2; DOCK10; LAMC1; SPTBN1; ARHGDIA; DOCK8; LGALS1; STC2; B2M; ECM1; LGALS3BP; SVEP1; BMP1; EEF1G; LMNA; TAGLN ; C14orf166; ENO1; LOX; TAGLN2; C1R; ENO1B; LTBP1; TGFBI; CALM1; ENO2; LUM; TGFBR3; CD109; FBLN1; MDH2; THBS1; CD44; FBN1; MRC2; TIMP1; CDH11; FBN2; MYH11; TIMP2; CDH13; FGF17; MYH9; TMSB4X; CDH2; FGFR2; NCL; TMSL3; CFH/HF1; FLJ21918; NPM1; TMSL6; CFL1; FLNA; PBP; TPI1; CLSTN1; FN1; PCOLCE; TPM4; COL11A1; FSTL1; PDGFRB; TUBA3; COL12A1; GALNT5; PFN1; TUBA6; COL16A1; GLRX; PGK1; TXN; COL1A1; GOLPH2; PGK2; TXNRD1; COL1A2; GSN; PLAU; UBE2I; COL3A1; GSTP1; PLEC1; UCHL1; COL4A1; HNRPCL1; PPIA; UFM1; COL4A2; HNRPF; PRDX1; URB; COL5A1; HNT; PRDX5; YWHAZ.

201 Genes in 58 Biological Processes

The particle may additionally, or alternatively, comprise any of the gene products of the 201 genes listed below in Table D3. These genes are characterised according to a biological process the gene is involved in.

Accordingly, the particle may be employed to affect, control or regulate any of these 58 biological processes.

Alcohol Metabolism	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Angiogenesis

GI_11321596-S KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)
GI_30172563-S VEGF	vascular endothelial growth factor
GI_28610153-S IL8	interleukin 8
GI_42716312-S ANG	angiogenin, ribonuclease, RNase A family, 5
GI_10337586-S FGF6	fibroblast growth factor 6

Biomineral Formation	
GI_27262662-A CDH11	cadherin 11, type 2, OB-cadherin (osteoblast)
GI_5902810-A BMP1	bone morphogenetic protein 1
GI_4507170-S SPARC	secreted protein, acidic, cysteine-rich (osteonectin)

Blood Vessel Devolopment	
GI_11321596-S KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)
GI_30172563-S VEGF	vascular endothelial growth factor
GI_28610153-S IL8	interleukin 8
GI_42716312-S ANG	angiogenin, ribonuclease, RNase A family, 5
GI_10337586-S FGF6	fibroblast growth factor 6

Blood Vessel Morphogenesis	
GI_11321596-S KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)
GI_30172563-S VEGF	vascular endothelial growth factor
GI_28610153-S IL8	interleukin 8
GI_42716312-S ANG	angiogenin, ribonuclease, RNase A family, 5
GI_10337586-S FGF6	fibroblast growth factor 6

Bone Remodeling	
GI_27262662-A CDH11	cadherin 11, type 2, OB-cadherin (osteoblast)
GI_5902810-A BMP1 BMP1	bone morphogenetic protein 1
GI_4507170-S SPARC	secreted protein, acidic, cysteine-rich (osteonectin)

Carbohydrate Metabolism	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Catabolism	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Cellular Carbohydrate Catabolism	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)

GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Cellular Carbohydrate Metabolism	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Cellular Catabolism	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Cellular Macromolecule Catabolism	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Chemotaxis	
GI_27262654-A IL16	interleukin 16 (lymphocyte chemoattractant factor)
GI_22538807-A CCL23	chemokine (C-C motif) ligand 23
GI_34335180-A CCL15	chemokine (C-C motif) ligand 15
GI_40316922-I CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
GI_41872613-S CXCL5	chemokine (C-X-C motif) ligand 5
GI_22538812-S CCL2	chemokine (C-C motif) ligand 2
GI_4504098-S CXCR3	chemokine (C-X-C motif) receptor 3
GI_22538399-S CCL11	chemokine (C-C motif) ligand 11
GI_5453576-S CXCL13	chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)
GI_22538815-S CCL8	chemokine (C-C motif) ligand 8
GI_34222286-S CYR61	cysteine-rich, angiogenic inducer, 61
GI_4506832-S CCL1	chemokine (C-C motif) ligand 1
GI_28610153-S IL8	interleukin 8
GI_22538800-S CCL16	chemokine (C-C motif) ligand 16
GI_14790145-S CXCL11	chemokine (C-X-C motif) ligand 11
GI_22165426-S CCL24	chemokine (C-C motif) ligand 24
GI_22538813-S CCL5	chemokine (C-C motif) ligand 5
GI_13435401-S CCL7	chemokine (C-C motif) ligand 7
GI_4505862-S PLAU	plasminogen activator, urokinase
GI_4504152-S CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating

	activity, alpha)
GI_27894329-S IL1A	interleukin 1, alpha
GI_22547151-S CCL26	chemokine (C-C motif) ligand 26
GI_4506852-S XCL1	chemokine (C motif) ligand 1

Defense Response	
GI_27894305-S IL1B	interleukin 1, beta
GI_27894329-S IL1A	interleukin 1, alpha

Development	
GI_42716312-S ANG	angiogenin, ribonuclease, RNase A family, 5
GI_5902810-A BMP1	bone morphogenetic protein 1
GI_27262662-A CDH11	cadherin 11, type 2, OB-cadherin (osteoblast)
GI_27262662-A CSF1	colony stimulating factor 1 (macrophage)
GI_27437029-S CSF2	colony stimulating factor 2 (granulocyte-macrophage)
GI_27437048-A CSF3	colony stimulating factor 3 (granulocyte)
GI_34222286-S CYR61	cysteine-rich, angiogenic inducer, 61
GI_24430140-S FBN1	fibrillin 1 (Marfan syndrome)
GI_4755135-S FBN2	fibrillin 2 (congenital contractural arachnodactyly)
GI_10337586-S FGF6	fibroblast growth factor 6
GI_24430216-S IL10	interleukin 10
GI_28610153-S IL8	interleukin 8
GI_11321596-S KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)
GI_4580419-A KITLG	KIT ligand
GI_6006018-S LIF	leukemia inhibitory factor (cholinergic differentiation factor)
GI_7262388-S PCOLCE	procollagen C-endopeptidase enhancer
GI_4507170-S SPARC	secreted protein, acidic, cysteine-rich (osteonectin)
GI_10863872-S TGFB1	transforming growth factor, beta 1 (Camurati-Engelmann disease)
GI_4507470-S TGFBR3	transforming growth factor, beta receptor III (betaglycan, 300kDa)
GI_40317625-S THBS1	thrombospondin 1
GI_40805871-S THPO	thrombopoietin (myeloproliferative leukemia virus oncogene ligand, megakaryocyte growth and development factor)
GI_4507508-S TIMP1	TIMP metallopeptidase inhibitor 1
GI_30172563-S VEGF	vascular endothelial growth factor

Energy Derivation by Oxidation of Organic Compounds	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Exocytosis	
GI_22538815-S CCL8	chemokine (C-C motif) ligand 8
GI_22538813-S CCL5	chemokine (C-C motif) ligand 5

Fever	
GI_27894305-S IL1B	interleukin 1, beta
GI_27894329-S IL1A	interleukin 1, alpha

Generation of Precursor Metabolites and Energy	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Glucose Catabolism	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Glucose Metabolism	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Glycolysis	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Heat Generation	
GI_27894305-S IL1B	interleukin 1, beta
GI_27894329-S IL1A	interleukin 1, alpha

Hemopoiesis	
GI_27262662-A CSF1	colony stimulating factor 1 (macrophage)
GI_4580419-A KITLG	KIT ligand
GI_24430216-S IL10	interleukin 10

Hemopoietic or Lymphoid Organ Development	
GI_27262662-A CSF1	colony stimulating factor 1 (macrophage)
GI_4580419-A KITLG	KIT ligand
GI_24430216-S IL10	interleukin 10

Hexose Catabolism	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)

GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Hexose Metabolism	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Immune Response	
GI_27894305-S IL1B	interleukin 1, beta
GI_27894329-S IL1A	interleukin 1, alpha

Inflammatory Response	
GI_27894305-S IL1B	interleukin 1, beta
GI_27894329-S IL1A	interleukin 1, alpha

Macromolecule Metabolism	
GI_5902810-A BMP1	bone morphogenetic protein 1
GI_31542249-S C1R	complement component 1, r subcomponent
GI_22538429-A CTSB	cathepsin B
GI_16507965-S ENO1	enolase 1, (alpha)
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_33859834-S HGF	hepatocyte growth factor (hepapoietin A; scatter factor)
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_13027798-S MMP1	matrix metallopeptidase 1 (interstitial collagenase)
GI_4505204-S MMP10	matrix metallopeptidase 10 (stromelysin 2)
GI_13027796-S MMP13	matrix metallopeptidase 13 (collagenase 3)
GI_13027803-S MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)
GI_4826835-S MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_4505862-S PLAU	plasminogen activator, urokinase
GI_26024330-S TPI1	triosephosphate isomerase 1

Macromolecule Catabolism	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Main Pathways of Carbohydrate Metabolism	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)

GI_16507965-S ENO1	enolase 1, (alpha)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Monosaccharide Catabolism	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Monosaccharide Metabolism	
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_16507965-S ENO1	enolase 1, (alpha)
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1

Organ Development	
GI_27262662-A CSF1	colony stimulating factor 1 (macrophage)
GI_27262662-A CDH11	cadherin 11, type 2, OB-cadherin (osteoblast)
GI_4580419-A KITLG	KIT ligand
GI_5902810-A BMP1	bone morphogenetic protein 1
GI_11321596-S KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)
GI_30172563-S VEGF	vascular endothelial growth factor
GI_28610153-S IL8	interleukin 8
GI_4507170-S SPARC	secreted protein, acidic, cysteine-rich (osteonectin)
GI_42716312-S ANG	angiogenin, ribonuclease, RNase A family, 5
GI_24430216-S IL10	interleukin 10
GI_10337586-S FGF6	fibroblast growth factor 6

Organ Morphogenesis	
GI_11321596-S KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)
GI_30172563-S VEGF	vascular endothelial growth factor
GI_28610153-S IL8	interleukin 8
GI_42716312-S ANG	angiogenin, ribonuclease, RNase A family, 5
GI_10337586-S FGF6	fibroblast growth factor 6

Ossification	
GI_27262662-A CDH11	cadherin 11, type 2, OB-cadherin (osteoblast)
GI_5902810-A BMP1	bone morphogenetic protein 1
GI_4507170-S SPARC	secreted protein, acidic, cysteine-rich (osteonectin)

Phosphate Metabolism	
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2

Phosphorus Metabolism	
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2

Response to Abiotic Stimulus	
GI_27262654-A IL16	interleukin 16 (lymphocyte chemoattractant factor)
GI_22538807-A CCL23	chemokine (C-C motif) ligand 23
GI_34335180-A CCL15	chemokine (C-C motif) ligand 15
GI_40316922-I CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
GI_41872613-S CXCL5	chemokine (C-X-C motif) ligand 5
GI_22538812-S CCL2	chemokine (C-C motif) ligand 2
GI_4504098-S CXCR3	chemokine (C-X-C motif) receptor 3
GI_22538399-S CCL11	chemokine (C-C motif) ligand 11
GI_5453576-S CXCL13	chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)
GI_22538815-S CCL8	chemokine (C-C motif) ligand 8
GI_34222286-S CYR61	cysteine-rich, angiogenic inducer, 61
GI_4506832-S CCL1	chemokine (C-C motif) ligand 1
GI_28610153-S IL8	interleukin 8
GI_22538800-S CCL16	chemokine (C-C motif) ligand 16
GI_14790145-S CXCL11	chemokine (C-X-C motif) ligand 11
GI_22165426-S CCL24	chemokine (C-C motif) ligand 24
GI_22538813-S CCL5	chemokine (C-C motif) ligand 5
GI_13435401-S CCL7	chemokine (C-C motif) ligand 7
GI_4505862-S PLAU	plasminogen activator, urokinase
GI_4504152-S CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
GI_27894329-S IL1A	interleukin 1, alpha
GI_22547151-S CCL26	chemokine (C-C motif) ligand 26
GI_4506852-S XCL1	chemokine (C motif) ligand 1

Response to Biotic Stimulus	
GI_27894305-S IL1B	interleukin 1, beta
GI_27894329-S IL1A	interleukin 1, alpha

Response to Chemical Stimulus	
GI_27262654-A IL16	interleukin 16 (lymphocyte chemoattractant factor)
GI_22538807-A CCL23	chemokine (C-C motif) ligand 23
GI_34335180-A CCL15	chemokine (C-C motif) ligand 15
GI_40316922-I CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
GI_41872613-S CXCL5	chemokine (C-X-C motif) ligand 5
GI_22538812-S CCL2	chemokine (C-C motif) ligand 2
GI_4504098-S CXCR3	chemokine (C-X-C motif) receptor 3
GI_22538399-S CCL11	chemokine (C-C motif) ligand 11
GI_5453576-S CXCL13	chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)
GI_22538815-S CCL8	chemokine (C-C motif) ligand 8
GI_34222286-S CYR61	cysteine-rich, angiogenic inducer, 61
GI_4506832-S CCL1	chemokine (C-C motif) ligand 1
GI_28610153-S IL8	interleukin 8
GI_22538800-S CCL16	chemokine (C-C motif) ligand 16

GI_14790145-S CXCL11	chemokine (C-X-C motif) ligand 11
GI_22165426-S CCL24	chemokine (C-C motif) ligand 24
GI_22538813-S CCL5	chemokine (C-C motif) ligand 5
GI_13435401-S CCL7	chemokine (C-C motif) ligand 7
GI_4505862-S PLAU	plasminogen activator, urokinase
GI_4504152-S CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
GI_27894329-S IL1A	interleukin 1, alpha
GI_22547151-S CCL26	chemokine (C-C motif) ligand 26
GI_4506852-S XCL1	chemokine (C motif) ligand 1

Response to External Biotic Stimulus	
GI_27894305-S IL1B	interleukin 1, beta
GI_27894329-S IL1A	interleukin 1, alpha

Response to External Stimulus	
GI_27262654-A IL16	interleukin 16 (lymphocyte chemoattractant factor)
GI_22538807-A CCL23	chemokine (C-C motif) ligand 23
GI_34335180-A CCL15	chemokine (C-C motif) ligand 15
GI_40316922-I CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
GI_41872613-S CXCL5	chemokine (C-X-C motif) ligand 5
GI_22538812-S CCL2	chemokine (C-C motif) ligand 2
GI_4504098-S CXCR3	chemokine (C-X-C motif) receptor 3
GI_22538399-S CCL11	chemokine (C-C motif) ligand 11
GI_5453576-S CXCL13	chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)
GI_22538815-S CCL8	chemokine (C-C motif) ligand 8
GI_34222286-S CYR61	cysteine-rich, angiogenic inducer, 61
GI_4506832-S CCL1	chemokine (C-C motif) ligand 1
GI_28610153-S IL8	interleukin 8
GI_22538800-S CCL16	chemokine (C-C motif) ligand 16
GI_14790145-S CXCL11	chemokine (C-X-C motif) ligand 11
GI_22165426-S CCL24	chemokine (C-C motif) ligand 24
GI_22538813-S CCL5	chemokine (C-C motif) ligand 5
GI_27894305-S IL1B	interleukin 1, beta
GI_13435401-S CCL7	chemokine (C-C motif) ligand 7
GI_4505862-S PLAU	plasminogen activator, urokinase
GI_4504152-S CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
GI_27894329-S IL1A	interleukin 1, alpha
GI_22547151-S CCL26	chemokine (C-C motif) ligand 26
GI_4506852-S XCL1	chemokine (C motif) ligand 1

Response to Pest, Pathogen or Parasite	
GI_27894305-S IL1B	interleukin 1, beta
GI_27894329-S IL1A	interleukin 1, alpha

Response to Stimulus	
GI_27262654-A IL16	interleukin 16 (lymphocyte chemoattractant factor)
GI_22538807-A CCL23	chemokine (C-C motif) ligand 23
GI_34335180-A CCL15	chemokine (C-C motif) ligand 15
GI_40316922-I CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)

GI_41872613-S CXCL5	chemokine (C-X-C motif) ligand 5
GI_22538812-S CCL2	chemokine (C-C motif) ligand 2
GI_4504098-S CXCR3	chemokine (C-X-C motif) receptor 3
GI_22538399-S CCL11	chemokine (C-C motif) ligand 11
GI_5453576-S CXCL13	chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)
GI_22538815-S CCL8	chemokine (C-C motif) ligand 8
GI_34222286-S CYR61	cysteine-rich, angiogenic inducer, 61
GI_4506832-S CCL1	chemokine (C-C motif) ligand 1
GI_28610153-S IL8	interleukin 8
GI_22538800-S CCL16	chemokine (C-C motif) ligand 16
GI_14790145-S CXCL11	chemokine (C-X-C motif) ligand 11
GI_22165426-S CCL24	chemokine (C-C motif) ligand 24
GI_22538813-S CCL5	chemokine (C-C motif) ligand 5
GI_27894305-S IL1B	interleukin 1, beta
GI_13435401-S CCL7	chemokine (C-C motif) ligand 7
GI_4505862-S PLAU	plasminogen activator, urokinase
GI_4504152-S CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
GI_27894329-S IL1A	interleukin 1, alpha
GI_22547151-S CCL26	chemokine (C-C motif) ligand 26
GI_4506852-S XCL1	chemokine (C motif) ligand 1

Response to Stress	
GI_27894305-S IL1B	interleukin 1, beta
GI_4505862-S PLAU	plasminogen activator, urokinase
GI_27894329-S IL1A	interleukin 1, alpha

Response to Wounding	
GI_27894305-S IL1B	interleukin 1, beta
GI_4505862-S PLAU	plasminogen activator, urokinase
GI_27894329-S IL1A	interleukin 1, alpha

Secretion	
GI_22538815-S CCL8	chemokine (C-C motif) ligand 8
GI_22538813-S CCL5	chemokine (C-C motif) ligand 5

Secretory Pathway	
GI_22538815-S CCL8	chemokine (C-C motif) ligand 8
GI_22538813-S CCL5	chemokine (C-C motif) ligand 5

Skeletal Development	
GI_27262662-A CDH11	cadherin 11, type 2, OB-cadherin (osteoblast)
GI_5902810-A BMP1 BMP1	bone morphogenetic protein 1
GI_4507170-S SPARC	secreted protein, acidic, cysteine-rich (osteonectin)

Taxis	
GI_27262654-A IL16	interleukin 16 (lymphocyte chemoattractant factor)
GI_22538807-A CCL23	chemokine (C-C motif) ligand 23
GI_34335180-A CCL15	chemokine (C-C motif) ligand 15
GI_40316922-I CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)

GI_41872613-S CXCL5	chemokine (C-X-C motif) ligand 5
GI_22538812-S CCL2	chemokine (C-C motif) ligand 2
GI_4504098-S CXCR3	chemokine (C-X-C motif) receptor 3
GI_22538399-S CCL11	chemokine (C-C motif) ligand 11
GI_5453576-S CXCL13	chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)
GI_22538815-S CCL8	chemokine (C-C motif) ligand 8
GI_34222286-S CYR61	cysteine-rich, angiogenic inducer, 61
GI_4506832-S CCL1	chemokine (C-C motif) ligand 1
GI_28610153-S IL8	interleukin 8
GI_22538800-S CCL16	chemokine (C-C motif) ligand 16
GI_14790145-S CXCL11	chemokine (C-X-C motif) ligand 11
GI_22165426-S CCL24	chemokine (C-C motif) ligand 24
GI_22538813-S CCL5	chemokine (C-C motif) ligand 5
GI_13435401-S CCL7	chemokine (C-C motif) ligand 7
GI_4505862-S PLAU	plasminogen activator, urokinase
GI_4504152-S CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
GI_27894329-S IL1A	interleukin 1, alpha
GI_22547151-S CCL26	chemokine (C-C motif) ligand 26
GI_4506852-S XCL1	chemokine (C motif) ligand 1

Thermoregulation	
GI_27894305-S IL1B	interleukin 1, beta
GI_27894329-S IL1A	interleukin 1, alpha

Tissue Development	
GI_27262662-A CDH11	cadherin 11, type 2, OB-cadherin (osteoblast)
GI_5902810-A BMP1 BMP1	bone morphogenetic protein 1
GI_4507170-S SPARC	secreted protein, acidic, cysteine-rich (osteonectin)

Vasulogenesis	
GI_30172563-S VEGF	vascular endothelial growth factor

Vasculature Development	
GI_11321596-S KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)
GI_30172563-S VEGF	vascular endothelial growth factor
GI_28610153-S IL8	interleukin 8
GI_42716312-S ANG	angiogenin, ribonuclease, RNase A family, 5
GI_10337586-S FGF6	fibroblast growth factor 6

Table D3. Listing of 201 Genes in each of the 58 Biological Processes (Genes of Mesenchymal Stem Cell Conditioned Medium (MSC-CM))

201 Genes in 30 Pathways

The particle may additionally, or alternatively, comprise any of the gene products of the 201 genes listed below in Table D4. These genes are characterised according to a pathway the gene is involved in.

Accordingly, the particle may be employed to affect, control or regulate any of these 30 pathways.

Antigen processing and presentation - Homo sapiens (human)	
GI_22538429-A CTSB	cathepsin B
GI_37704380-S B2M	beta-2-microglobulin
GI_24234685-A HSPA8	heat shock 70kDa protein 8
GI_30581139-A PSME1	proteasome (prosome, macropain) activator subunit 1 (PA28 alpha)
Apoptosis - Homo sapiens (human)	
GI_41281560-S CLSTN1	calsyntenin 1
GI_28416914-S IL3	interleukin 3 (colony-stimulating factor, multiple)
GI_27894329-S IL1A	interleukin 1, alpha
GI_27894305-S IL1B	interleukin 1, beta
GI_25952110-S TNF	tumor necrosis factor (TNF superfamily, member 2)
Carbon fixation - Homo sapiens (human)	
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
GI_26024330-S TPI1	triosephosphate isomerase 1
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
Cell Communication - Homo sapiens (human)	
GI_27436944-A LMNA	lamin A/C
GI_5016088-S ACTB	actin, beta
GI_10938011-S ACTC	actin, alpha, cardiac muscle
GI_11038618-S ACTG1	actin, gamma 1
GI_14719826-S COL1A1	collagen, type I, alpha 1
GI_21536289-S COL1A2	collagen, type I, alpha 2
GI_15149480-S COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)
GI_45580690-S COL4A1	collagen, type IV, alpha 1
GI_17986276-S COL4A2	collagen, type IV, alpha 2
GI_16554578-S COL5A1	collagen, type V, alpha 1
GI_16554580-S COL5A2	collagen, type V, alpha 2
GI_15011912-S COL6A1	collagen, type VI, alpha 1
GI_17402876-A COL6A2	collagen, type VI, alpha 2
GI_17149810-A COL6A3	collagen, type VI, alpha 3
GI_18375521-A COL11A1	collagen, type XI, alpha 1
GI_16933543-A FN1	fibronectin 1
GI_9845497-S LAMC1	laminin, gamma 1 (formerly LAMB2)
GI_16554581-S COL5A3	collagen, type V, alpha 3
GI_40317625-S THBS1	thrombospondin 1
Citrate cycle (TCA cycle) - Homo sapiens (human)	
GI_4504374-S CFH1/HF1	complement factor H
GI_21735620-S MDH2	malate dehydrogenase 2, NAD (mitochondrial)
Complement and coagulation cascades - Homo sapiens (human)	
GI_4505862-S PLAU	plasminogen activator, urokinase
GI_4504374-S CFH1/HF1	complement factor H
GI_31542249-S C1R	complement component 1, r subcomponent
Cytokine-cytokine receptor interaction - Homo sapiens (human)	
GI_4506832-S CCL1	chemokine (C-C motif) ligand 1

GI_22538399-S CCL11	chemokine (C-C motif) ligand 11
GI_34335180-A CCL15	chemokine (C-C motif) ligand 15
GI_22538800-S CCL16	chemokine (C-C motif) ligand 16
GI_22538812-S CCL2	chemokine (C-C motif) ligand 2
GI_22538807-A CCL23	chemokine (C-C motif) ligand 23
GI_22165426-S CCL24	chemokine (C-C motif) ligand 24
GI_22547151-S CCL26	chemokine (C-C motif) ligand 26
GI_22538813-S CCL5	chemokine (C-C motif) ligand 5
GI_13435401-S CCL7	chemokine (C-C motif) ligand 7
GI_22538815-S CCL8	chemokine (C-C motif) ligand 8
GI_27262662-A CSF1	colony stimulating factor 1 (macrophage)
GI_27437029-S CSF2	colony stimulating factor 2 (granulocyte-macrophage)
GI_27437048-A CSF3	colony stimulating factor 3 (granulocyte)
GI_4506856-S CX3CL1	chemokine (C-X3-C motif) ligand 1
GI_4504152-S CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
GI_14790145-S CXCL11	chemokine (C-X-C motif) ligand 11
GI_40316922-I CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
GI_5453576-S CXCL13	chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)
GI_41872613-S CXCL5	chemokine (C-X-C motif) ligand 5
GI_4504098-S CXCR3	chemokine (C-X-C motif) receptor 3
GI_6031163-S EGF	epidermal growth factor (beta-urogastrone)
GI_38455415-S FLT3LG	fms-related tyrosine kinase 3 ligand
GI_33859834-S HGF	hepatocyte growth factor (hepapoietin A; scatter factor)
GI_10835170-S IFNG	interferon, gamma
GI_24430216-S IL10	interleukin 10
GI_24497437-S IL12B	interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)
GI_26787977-S IL13	interleukin 13
GI_27894329-S IL1A	interleukin 1, alpha
GI_27894305-S IL1B	interleukin 1, beta
GI_28178860-S IL2	interleukin 2
GI_28416914-S IL3	interleukin 3 (colony-stimulating factor, multiple)
GI_10834983-S IL6	interleukin 6 (interferon, beta 2)
GI_28610152-S IL7	interleukin 7
GI_28610153-S IL8	interleukin 8
GI_11321596-S KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)
GI_4580419-A KITLG	KIT ligand
GI_4557714-S LEP	leptin (obesity homolog, mouse)
GI_6006018-S LIF	leukemia inhibitory factor (cholinergic differentiation factor)
GI_15451785-A PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)
GI_15451788-S PDGFRB	platelet-derived growth factor receptor, beta polypeptide
GI_10863872-S TGFB1	transforming growth factor, beta 1 (Camurati-Engelmann disease)
GI_4507462-S TGFB2	transforming growth factor, beta 2
GI_40805871-S THPO	thrombopoietin (myeloproliferative leukemia virus oncogene ligand, megakaryocyte growth and development factor)
GI_4507508-S TIMP1	TIMP metallopeptidase inhibitor 1
GI_25952110-S TNF	tumor necrosis factor (TNF superfamily, member 2)
GI_22547122-S TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)
GI_30172563-S VEGF	vascular endothelial growth factor
GI_4506852-S XCL1	chemokine (C motif) ligand 1
ECM-receptor interaction - Homo sapiens (human)	
GI_9845497-S LAMC1	laminin, gamma 1 (formerly LAMB2)

GI_14719826-S COL1A1	collagen, type I, alpha 1
GI_21536289-S COL1A2	collagen, type I, alpha 2
GI_15149480-S COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)
GI_45580690-S COL4A1	collagen, type IV, alpha 1
GI_17986276-S COL4A2	collagen, type IV, alpha 2
GI_16554578-S COL5A1	collagen, type V, alpha 1
GI_16554580-S COL5A2	collagen, type V, alpha 2
GI_15011912-S COL6A1	collagen, type VI, alpha 1
GI_17402876-A COL6A2	collagen, type VI, alpha 2
GI_17149810-A COL6A3	collagen, type VI, alpha 3
GI_18375521-A COL11A1	collagen, type XI, alpha 1
GI_16554581-S COL5A3	collagen, type V, alpha 3
GI_16933543-A FN1	fibronectin 1
GI_40317625-S THBS1	thrombospondin 1
GI_7427516-S HSPG2	heparan sulfate proteoglycan 2 (perlecan)
GI_21361192-S CD44	CD44 antigen (homing function and Indian blood group system)
Epithelial cell signaling in Helicobacter pylori infection - Homo sapiens (human)	
GI_22538813-S CCL5	chemokine (C-C motif) ligand 5
GI_4504152-S CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
GI_28610153-S IL8	interleukin 8
Fc epsilon RI signaling pathway - Homo sapiens (human)	
GI_25952110-S TNF	tumor necrosis factor (TNF superfamily, member 2)
GI_26787977-S IL13	interleukin 13
GI_27437029-S CSF2	colony stimulating factor 2 (granulocyte-macrophage)
GI_41281560-S CLSTN1	calsyntenin 1
GI_28416914-S IL3	interleukin 3 (colony-stimulating factor, multiple)
Focal adhesion - Homo sapiens (human)	
GI_5016088-S ACTB	actin, beta
GI_10938011-S ACTC	actin, alpha, cardiac muscle
GI_11038618-S ACTG1	actin, gamma 1
GI_41281560-S CLSTN1	calsyntenin 1
GI_18375521-A COL11A1	collagen, type XI, alpha 1
GI_14719826-S COL1A1	collagen, type I, alpha 1
GI_21536289-S COL1A2	collagen, type I, alpha 2
GI_15149480-S COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)
GI_45580690-S COL4A1	collagen, type IV, alpha 1
GI_17986276-S COL4A2	collagen, type IV, alpha 2
GI_16554578-S COL5A1	collagen, type V, alpha 1
GI_16554580-S COL5A2	collagen, type V, alpha 2
GI_16554581-S COL5A3	collagen, type V, alpha 3
GI_15011912-S COL6A1	collagen, type VI, alpha 1
GI_17402876-A COL6A2	collagen, type VI, alpha 2
GI_17149810-A COL6A3	collagen, type VI, alpha 3
GI_6031163-S EGF	epidermal growth factor (beta-urogastrone)
GI_4503744-S FLNA	filamin A, alpha (actin binding protein 280)
GI_16933543-A FN1	fibronectin 1
GI_33859834-S HGF	hepatocyte growth factor (hepapoietin A; scatter factor)
GI_19923111-S IGF1	insulin-like growth factor 1 (somatomedin C)
GI_10834983-S IL6	interleukin 6 (interferon, beta 2)

GI_11321596-S KDR	kinase insert domain receptor (a type III receptor tyrosine kinase)
GI_9845497-S LAMC1	laminin, gamma 1 (formerly LAMB2)
GI_15451785-A PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)
GI_15451788-S PDGFRB	platelet-derived growth factor receptor, beta polypeptide
GI_40317625-S THBS1	thrombospondin 1
GI_30172563-S VEGF	vascular endothelial growth factor
Gap junction - Homo sapiens (human)	
GI_5174476-S K-ALPHA-1	tubulin, alpha, ubiquitous
GI_17986282-S TUBA3	tubulin, alpha 3
GI_31880337-S TUBA6	tubulin, alpha 6
GI_15451788-S PDGFRB	platelet-derived growth factor receptor, beta polypeptide
GI_6031163-S EGF	epidermal growth factor (beta-urogastrone)
GI_15451785-A PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)
Glycolysis or Gluconeogenesis - Homo sapiens (human)	
GI_16507965-S ENO1	enolase 1, (alpha)
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
GI_26024330-S TPI1	triosephosphate isomerase 1
GI_22095338-S PGK1	phosphoglycerate kinase 1
GI_31543396-S PGK2	phosphoglycerate kinase 2
Hematopoietic cell lineage - Homo sapiens (human)	
GI_21361192-S CD44	CD44 antigen (homing function and Indian blood group system)
GI_4503744-S FLNA	filamin A, alpha (actin binding protein 280)
GI_40805871-S THPO	thrombopoietin (myeloproliferative leukemia virus oncogene ligand, megakaryocyte growth and development factor)
GI_4507508-S TIMP1	TIMP metallopeptidase inhibitor 1
GI_25952110-S TNF	tumor necrosis factor (TNF superfamily, member 2)
GI_27262662-A CSF1	colony stimulating factor 1 (macrophage)
GI_27437048-A CSF3	colony stimulating factor 3 (granulocyte)
GI_38455415-S FLT3LG	fms-related tyrosine kinase 3 ligand
GI_10834983-S IL6	interleukin 6 (interferon, beta 2)
GI_27437029-S CSF2	colony stimulating factor 2 (granulocyte-macrophage)
GI_27894329-S IL1A	interleukin 1, alpha
GI_27894305-S IL1B	interleukin 1, beta
GI_28416914-S IL3	interleukin 3 (colony-stimulating factor, multiple)
GI_28610152-S IL7	interleukin 7
GI_4580419-A KITLG	KIT ligand
Inositol metabolism - Homo sapiens (human)	
GI_26024330-S TPI1	triosephosphate isomerase 1
Insulin signaling pathway - Homo sapiens (human)	
GI_31377794-S CALM1	calmodulin 1 (phosphorylase kinase, delta)
GI_41281560-S CLSTN1	calsyntenin 1
Jak-STAT signaling pathway - Homo sapiens (human)	
GI_41281560-S CLSTN1	calsyntenin 1
GI_27437029-S CSF2	colony stimulating factor 2 (granulocyte-macrophage)
GI_27437048-A CSF3	colony stimulating factor 3 (granulocyte)
GI_10835170-S IFNG	interferon, gamma

GI_24430216-S IL10	interleukin 10
GI_24497437-S IL12B	interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)
GI_26787977-S IL13	interleukin 13
GI_28178860-S IL2	interleukin 2
GI_28416914-S IL3	interleukin 3 (colony-stimulating factor, multiple)
GI_10834983-S IL6	interleukin 6 (interferon, beta 2)
GI_28610152-S IL7	interleukin 7
GI_4557714-S LEP	leptin (obesity homolog, mouse)
GI_6006018-S LIF	leukemia inhibitory factor (cholinergic differentiation factor)
GI_40805871-S THPO	thrombopoietin (myeloproliferative leukemia virus oncogene ligand, megakaryocyte growth and development factor)
GI_4507508-S TIMP1	TIMP metallopeptidase inhibitor 1
Leukocyte transendothelial migration - Homo sapiens (human)	
GI_4826835-S MMP9	matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
GI_5453576-S CXCL13	chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)
GI_28610153-S IL8	interleukin 8
GI_40316922-I CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
GI_41281560-S CLSTN1	calsyntenin 1
GI_5016088-S ACTB	actin, beta
GI_10938011-S ACTC	actin, alpha, cardiac muscle
GI_11038618-S ACTG1	actin, gamma 1
MAPK signaling pathway - Homo sapiens (human)	
GI_34106709-A BDNF	brain-derived neurotrophic factor
GI_6031163-S EGF	epidermal growth factor (beta-urogastrone)
GI_4503692-S FGF17	fibroblast growth factor 17
GI_4503700-S FGF4	fibroblast growth factor 4 (heparin secretory transforming protein 1, Kaposi sarcoma oncogene)
GI_10337586-S FGF6	fibroblast growth factor 6
GI_15147344-S FGF7	fibroblast growth factor 7 (keratinocyte growth factor)
GI_4503706-S FGF9	fibroblast growth factor 9 (glia-activating factor)
GI_13186266-A FGFR2	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)
GI_4503744-S FLNA	filamin A, alpha (actin binding protein 280)
GI_40549401-A GDNF	glial cell derived neurotrophic factor
GI_24234685-A HSPA8	heat shock 70kDa protein 8
GI_27894329-S IL1A	interleukin 1, alpha
GI_27894305-S IL1B	interleukin 1, beta
GI_15451785-A PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)
GI_15451788-S PDGFRB	platelet-derived growth factor receptor, beta polypeptide
GI_10863872-S TGFB1	transforming growth factor, beta 1 (Camurati-Engelmann disease)
GI_4507462-S TGFB2	transforming growth factor, beta 2
GI_25952110-S TNF	tumor necrosis factor (TNF superfamily, member 2)
Methane metabolism - Homo sapiens (human)	
GI_40805871-S THPO	thrombopoietin (myeloproliferative leukemia virus oncogene ligand, megakaryocyte growth and development factor)
GI_32455261-A PRDX5	peroxiredoxin 5
mTOR signaling pathway - Homo sapiens (human)	

GI_30172563-S VEGF	vascular endothelial growth factor
GI_41281560-S CLSTN1	calsyntenin 1
GI_19923111-S IGF1	insulin-like growth factor 1 (somatomedin C)
Phenylalanine, tyrosine and tryptophan biosynthesis - Homo sapiens (human)	
GI_16507965-S ENO1	enolase 1, (alpha)
GI_16507966-S ENO2	enolase 2 (gamma, neuronal)
Phenylalanine metabolism - Homo sapiens (human)	
GI_4505184-S MIF	macrophage migration inhibitory factor (glycosylation-inhibiting factor)
GI_40805871-S THPO	thrombopoietin (myeloproliferative leukemia virus oncogene ligand, megakaryocyte growth and development factor)
GI_32455261-A PRDX5	peroxiredoxin 5
Regulation of actin cytoskeleton - Homo sapiens (human)	
GI_5016088-S ACTB	actin, beta
GI_10938011-S ACTC	actin, alpha, cardiac muscle
GI_11038618-S ACTG1	actin, gamma 1
GI_5031634-S CFL1	cofilin 1 (non-muscle)
GI_41281560-S CLSTN1	calsyntenin 1
GI_6031163-S EGF	epidermal growth factor (beta-urogastrone)
GI_4503692-S FGF17	fibroblast growth factor 17
GI_4503700-S FGF4	fibroblast growth factor 4 (heparin secretory transforming protein 1, Kaposi sarcoma oncogene)
GI_10337586-S FGF6	fibroblast growth factor 6
GI_15147344-S FGF7	fibroblast growth factor 7 (keratinocyte growth factor)
GI_4503706-S FGF9	fibroblast growth factor 9 (glia-activating factor)
GI_13186266-A FGFR2	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)
GI_16933543-A FN1	fibronectin 1
GI_38044287-A GSN	gelsolin (amyloidosis, Finnish type)
GI_22507396-S MYH9	myosin, heavy polypeptide 9, non-muscle
GI_15451785-A PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)
GI_15451788-S PDGFRB	platelet-derived growth factor receptor, beta polypeptide
GI_16753213-S PFN1	profilin 1
GI_34328943-S TMSB4X	thymosin, beta 4, X-linked
GI_34013529-S TMSL3	thymosin-like 3
Stilbene, coumarine and lignin biosynthesis - Homo sapiens (human)	
GI_40805871-S THPO	thrombopoietin (myeloproliferative leukemia virus oncogene ligand, megakaryocyte growth and development factor)
GI_32455261-A PRDX5	peroxiredoxin 5
T cell receptor signaling pathway - Homo sapiens (human)	
GI_25952110-S TNF	tumor necrosis factor (TNF superfamily, member 2)
GI_27437029-S CSF2	colony stimulating factor 2 (granulocyte-macrophage)
GI_10835170-S IFNG	interferon, gamma
GI_24430216-S IL10	interleukin 10
GI_28178860-S IL2	interleukin 2
GI_41281560-S CLSTN1	calsyntenin 1
TGF-beta signaling pathway - Homo sapiens (human)	
GI_4557730-S LTBP1	latent transforming growth factor beta binding protein 1
GI_40317625-S THBS1	thrombospondin 1

GI_10863872-S TGFB1	transforming growth factor, beta 1 (Camurati-Engelmann disease)
GI_4507462-S TGFB2	transforming growth factor, beta 2
GI_25952110-S TNF	tumor necrosis factor (TNF superfamily, member 2)
GI_10835170-S IFNG	interferon, gamma
Tight junction - Homo sapiens (human)	
GI_5016088-S ACTB	actin, beta
GI_10938011-S ACTC	actin, alpha, cardiac muscle
GI_11038618-S ACTG1	actin, gamma 1
GI_22507396-S MYH9	myosin, heavy polypeptide 9, non-muscle
Toll-like receptor signaling pathway - Homo sapiens (human)	
GI_14790145-S CXCL11	chemokine (C-X-C motif) ligand 11
GI_28610153-S IL8	interleukin 8
GI_24497437-S IL12B	interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)
GI_27894305-S IL1B	interleukin 1, beta
GI_41281560-S CLSTN1	calsyntenin 1
GI_10834983-S IL6	interleukin 6 (interferon, beta 2)
GI_22538813-S CCL5	chemokine (C-C motif) ligand 5
GI_25952110-S TNF	tumor necrosis factor (TNF superfamily, member 2)
Type I diabetes mellitus - Homo sapiens (human)	
GI_24497437-S IL12B	interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)
GI_28178860-S IL2	interleukin 2
GI_10835170-S IFNG	interferon, gamma
GI_27894329-S IL1A	interleukin 1, alpha
GI_27894305-S IL1B	interleukin 1, beta
GI_25952110-S TNF	tumor necrosis factor (TNF superfamily, member 2)

Table D4. Listing of 201 Genes in Each of the 30 Pathways (Genes of Mesenchymal Stem Cell Conditioned Medium (MSC-CM))

593 Genes and 794 Gene Products

The particle may additionally, or alternatively, comprise any of the gene products of the 593 genes and/or 794 gene products listed below in Table D5.

Accordingly, the particle may be employed to affect, control or regulate any of the biological processes or pathways the genes or gene products are involved in.

Total 593 unique gene products: set 1-4 (3 of 4 & 4 of 4) without common genes in 201 list							
02-Sep	CAND1	DDX17	H2AFY	KRTHB4	PCBP2	QARS	STC1
07-Sep	CAP1	DES	HADH	LAMA4	PCDH18	QPCT	STIP1
AARS	CAP2	DLD	HARS	LAMB1	PCDHGB6	RAB11B	SULF1
ACAA2	CAPG	DNAJC3	HARS2	LANCL1	PCK2	RAB1A	SYNCRIP
ACAT2	CAPN1	DPP3	hCG_1641617	LAP3	PCMT1	RAB6A	TALDO1
ACO1	CAPN2	DPYSL2	hCG_2023776	LASP1	PCNA	RAC1	TARS
ACTN1	CAPZA1	DPYSL3	HEXA	LDHA	PDCD6IP	RAN	TCN2
ACTN2	CAPZA2	DSTN	HEXB	LDHAL6B	PDGFC	RANBP5	TCP1
ACTN3	CAPZB	DYNLL1	HIBCH	LDHB	PDIA3	RARRES2	TFPI
ACTN4	CARS	ECHS1	HINT1	LEPRE1	PDIA4	RARS	THBS2
ACTR1A	CBR1	EEF1A1	HIST1H4	LGALS3	PDIA6	RBMX	THOP1
ACTR1B	CBR3	EEF1A2	HIST1H4A	LOC196463	PDLIM1	RHOA	THY1
ACTR2	CCBL2	EEF1B2	HIST1H4B	LOC283523	PDLIM5	RNASE4	TKT

ACTR3	CCDC19	EEF2	HIST1H4C	LOC347701	PDLIM7	RNH1	TLN1
ACTR3B	CCT2	EFEMP2	HIST1H4D	LOC646821	PEPD	RNPEP	TMOD2
ADAM9	CCT3	EIF2S3	HIST1H4E	LOC649125	PFN2	RPL10A	TMOD3
ADSL	CCT4	EIF3S9	HIST1H4F	LOC653214	PGCP	RPL11	TNC
ADSS	CCT5	EIF4A1	HIST1H4H	LOC654188	PGD	RPL12	TNPO1
AEBP1	CCT6A	EIF4A2	HIST1H4I	LOC728378	PGLS	RPL14	TP53I3
AGA	CCT7	EMILIN1	HIST1H4J	LOXL2	PGM1	RPL18	TPM1
AHCY	CCT8	ENO3	HIST1H4K	LRP1	PGRMC2	RPL22	TPM2
AK1	CD248	EPPK1	HIST1H4L	LTA4H	PHGDH	RPL30	TPM3
AK2	CD59	EPRS	HIST2H2AA3	LTB4DH	PHPT1	RPL5	TRAP1
AKR1A1	CD81	ESD	HIST2H2AA4	LTBP2	PICALM	RPL7	TRHDE
AKR1B1	CD9	ETF1	HIST2H4A	M6PRBP1	PKM2	RPLP0	TROVE2
ALDH2	CDC37	ETFB	HIST2H4B	MACF1	PLEKHC1	RPLP1	TSKU
ALDH7A1	CDC42	ETHE1	HIST4H4	MAP1B	PLOD1	RPLP2	TUBA1A
ALDOA	CFL2	EXT1	HLA-A	MAPK1	PLOD2	RPS10	TUBA8
ALDOC	CHID1	FAH	HLA-B	MAPRE1	PLOD3	RPS15A	TUBB
ANXA1	CLEC11A	FAHD1	HMX1	MAT2A	PLS1	RPS16	TUBB2C
ANXA5	CLIC1	FAM129B	HNRPA1	MAT2B	PLS3	RPS19	TUBB3
ANXA6	CLIC4	FAM3C	HNRPA1L-2	MCTS1	PLSCR3	RPS2	TUBB4
AP1B1	CLTC	FAM49B	HNRPA2B1	MDH1	POSTN	RPS20	TUBB6
AP1S1	CLTCL1	FAM62A	HNRPC	MFAP4	PPCS	RPS23	TUBB8
AP2A1	CLU	FBLN5	HNRPD	MGAT5	PPIB	RPS3	TWF1
AP2A2	CMPK	FDPS	HNRPDL	MMP14	PPP2R1A	RPS4X	TXNL5
AP2B1	CNDP2	FH	HNRPH2	MMP2	PPP2R4	RPS5	UBE1
AP3B1	CNN2	FKBP10	HNRPK	MRLC2	PPP5C	RPS7	UBE2L3
APEX1	CNN3	FKBP1A	HNRPL	MSN	PPP6C	RPS8	UBE2N
API5	COL18A1	FKBP3	HNRPR	MTAP	PRDX2	RPS9	UBE2V1
APOA1BP	COL2A1	FLNB	HNRPU	MTPN	PRDX3	RPSA	UBE3B
APOE	COL4A2	FLNC	HSP90AB1	MVP	PRDX4	RSU1	UCHL3
APP	COL5A1	FLRT2	HSP90B1	MXRA5	PRDX6	S100A16	UGDH
APRT	COL5A2	FLT1	HSPA1A	MXRA8	PRG1	SARS	UGP2
ARCN1	COL6A2	FSCN1	HSPA1B	MYH14	PRKACA	SDC4	UROD
ARHGAP1	COL7A1	FSTL5	HSPA1L	MYL6	PRKCSH	SDCBP	USP14
ARPC1A	COL7A1	FTL	HSPA4	NAGK	PRNP	SEC22B	USP5
ARPC1B	COL7A1	G6PD	HSPA5	NANS	PROCR	SEC23A	VARS
ARPC2	COL7A1	GALNT2	HSPA6	NARS	PROSC	SEC31A	VASN
ARPC3	COPA	GANAB	HSPB1	NEDD8	PRSS23	SEMA3C	VAT1
ARPC4	COPG	GAPDH	HSPD1	NEFM	PRSS3	SEMA7A	VCL
ARTS-1	COPS3	GARS	HSPH1	NIT2	PSAT1	SERPINB1	VCP
ATIC	COPS4	GAS6	HTRA1	NME1	PSMA1	SERPINB6	VIL2
ATP5B	COPS8	GBA	IDH1	NPC2	PSMA2	SERPINE1	VIM
ATP6AP1	CORO1B	GBE1	IGFBP3	NPEPPS	PSMA3	SERPINE2	VPS26A
CORO1C	GDF15	IGKC	NQO1	PSMA6	SERPINF1	VTN	VPS35
COTL1	GDI1	ILF2	NRP1	PSMA7	SERPINH1	WARS	VTN
CRIP2	GDI2	ILF3	NRP2	PSMB1	SERPINI2	WDR1	WARS
CS	GLO1	INHBA	NT5E	PSMB2	SFRP1	WNT5A	WDR1
BASP1	CSE1L	GLT8D3	IQGAP1	NUCB1	PSMB3	SIL1	WNT5A
BAT1	CSRP1	GLUD1	ISOC1	OLFML3	PSMB4	SLC1A5	WNT5B
BBS1	CSRP2	GM2A	ITGA2	P4HA1	PSMB5	SLC3A2	XPO1
BCAT1	CST3	GNPDA1	ITGB4BP	P4HB	PSMD11	SND1	YKT6
BGN	CTGF	GNPNAT1	KPNB1	PABPC1	PSMD13	SNRPD1	YWHAB
BLVRA	CTHRC1	GOT1	KRT1	PABPC4	PSMD5	SNRPE	YWHAE
BPNT1	CTSD	GOT2	KRT14	PAFAH1B1	PSMD6	SPOCK	YWHAG
BTD	CTSZ	GPC1	KRT2	PAFAH1B2	PSMD7	SPTAN1	YWHAH
C14orf141	CYCS	GPI	KRT27	PAFAH1B3	PSME2	SPTBN4	YWHAQ
C19orf10	D4ST1	GREM1	KRT4	PAICS	PTBP1	SRP9	
C1orf58	DAG1	GRHPR	KRT5	PAM	PTPRCAP	SRPX	
C1orf78	DCI	GSR	KRT6L	PAPPA	PTX3	SRPX2	
C1QBP	DCN	GSS	KRT7	PARK7	PURA	SSB	
C1S	DDAH2	GSTK1	KRT75	PARP1	PXDN	ST13	
C21orf33	DDB1	GSTO1	KRT77	PARVA	PYCR1	ST6GAL2	
CALR	DDT	GTPBP9	KRT9	PCBP1	PYGB	STAT1	

Table D5. 593 Additional Proteins in Mesenchymal Stem Cell Conditioned Medium (MSC-CM)

EXOSOME

The particle may in particular comprise a vesicle. The particle may comprise an exosome.

The Examples describe the isolation of the active component in the secretion that confers cardioprotection against the reperfusion injury. The active component may comprise an exosome secreted by the mesenchymal stem cells (MSCs).

Exosomes are small membrane vesicles formed in late endocytic compartments (multivesicular bodies) first described to be secreted by reticulocytes in 1983 [21] and subsequently found to be secreted by many cells types including various haematopoietic cells, tumours of haematopoietic or non-haematopoietic origin and epithelial cells [22]. They are distinct entities from the more recently described 'ribonuclease complex' also named exosome [23].

Exosomes may be defined by morphological and biochemical parameters (see reviews [22, 24-35]). Accordingly, the particles described here may comprise one or more of these morphological or biochemical parameters.

Exosomes are classically defined as "saucer-like" vesicles or a flattened sphere limited by a lipid bilayer with diameters of 40-100 nm and are formed by inward budding of the endosomal membrane. Like all lipid vesicles and unlike protein aggregates or nucleosomal fragments that are released by apoptotic cells, exosomes have a density of ~1.13 – 1.19 g/ml and float on sucrose gradients. Exosomes are enriched in cholesterol and sphingomyelin, and lipid raft markers such as GM1, GM3, flotillin and the src protein kinase Lyn suggesting that their membranes are enriched in lipid rafts.

The molecular composition of exosomes from different cell types and of different species has been examined. In general, exosomes contain ubiquitous proteins that appear to be common to all exosomes and proteins that are cell-type specific. Also, proteins in exosomes from the same cell-type but of different species are highly conserved. The ubiquitous exosome-associated proteins include cytosolic proteins found in cytoskeleton e.g. tubulin, actin and actin-binding proteins, intracellular membrane fusions and transport e.g. annexins and rab proteins, signal transduction proteins e.g. protein kinases, 14-3-3 and heterotrimeric G proteins, metabolic enzymes e.g. peroxidases, pyruvate and lipid kinases,

and enolase-1 and the family of tetraspanins e.g. CD9, CD63, CD81 and CD82. The tetraspannins are highly enriched in exosomes and are known to be involved in the organization of large molecular complexes and membrane subdomains.

Examples of cell-type specific proteins in exosomes are MHC class II molecules in exosomes from MHC class II-expressing cells, CD86 in dendritic cell-derived exosomes, T-cell receptors on T-cell-derived exosomes etc. Notably, exosomes do not contain proteins of nuclear, mitochondrial, endoplasmic-reticulum or Golgi-apparatus origin. Also, highly abundant plasma membrane proteins are absent in exosomes suggesting that they are not simply fragments of the plasma membrane. Many of the reported ubiquitous exosome-associated proteins are also present in the proteomic profile of the hESC-MSC secretion.

Exosomes are also known to contain mRNA and microRNA, which can be delivered to another cell, and can be functional in this new location[36]. The physiological functions of exosome remain poorly defined. It is thought to help eradicate obsolete proteins, recycle proteins, mediate tramission of infectious particles such as prions and viruses, induce complement resistance, facilitate immune cell-cell communication and transmit cell signaling [1, 22, 25-28, 37-40]. Exosomes have been used in immunotherapy for treatment of cancer[34]

USES OF PARTICLES FROM MESENCHYMAL STEM CELLS

The particle may be used as a substitute for an MSC or MSC-CM, as described above In particular, the particle may be used for any of the therapeutic purposes that MSCs or MSC-CMs are currently being used, or in the future may be used.

It will be evident that the methods and compositions described here enable the production of particles from mesenchymal stem cells. Thus, any uses of mesenchymal stem cells will equally attach to particles from mesenchymal stem cells.

Mesenchymal stem cells and differentiated cells produced by the methods and compositions described here may be used for, or for the preparation of a pharmaceutical composition for, the treatment of a disease. Such disease may comprise a disease treatable by regenerative therapy, including cardiac failure, bone marrow disease, skin disease, burns, degenerative disease such as diabetes, Alzheimer's disease, Parkinson's disease, etc and cancer. Accordingly, particles from MSCs may be used to treat such diseases.

Particles from mesenchymal stem cells such as those made according to the methods and compositions described here may be used for a variety of commercially important research, diagnostic, and therapeutic purposes.

The particles from mesenchymal stem cells may in particular be used for the preparation of a pharmaceutical composition for the treatment of disease. Such disease may comprise a disease treatable by regenerative therapy, including cardiac failure, bone marrow disease, skin disease, burns, degenerative disease such as diabetes, Alzheimer's disease, Parkinson's disease, etc and cancer.

Mesenchymal stem cells made by the methods and compositions described here have similar or identical properties to bone marrow derived mesenchymal stem cells (BM-MSCs). Therefore, the mesenchymal stem cells, and any differentiated cells made from these, as well as particles derived therefrom, may be used in any of the applications for which BM-MSCs are known to be used, or in which it is possible for them to be used.

DISEASES TREATABLE BY PARTICLES FROM MESENCHYMAL STEM CELLS

Analysis of the proteome of MSCs shows that the proteins expressed are involved in three biological processes: metabolism, defense response, and tissue differentiation including vascularization, hematopoiesis and skeletal development. Accordingly, the particles from MSCs described here may be used to treat diseases which these functions may have a role in, or whose repair or treatment involves any one or more of these biological processes. Similarly, the proteins expressed by the MSCs, singly or in combination, preferably in the form of particles as described here, may be used to supplement the activity of, or in place of, the MSCs, or media conditioned by the MSCs, for the purpose of for example treating or preventing such diseases.

The 201 gene products expressed by the MSCs are shown to activate important signalling pathways in cardiovascular biology, bone development and hematopoiesis such as Jak-STAT, MAPK, Toll-like receptor, TGF-beta signalling and mTOR signaling pathways. Accordingly, the particles from the MSCs, etc, may be used to prevent or treat a disease in which any of these signalling pathways is involved, or whose aetiology involves one or more defects in any one or more of these signalling pathways.

Accordingly, such particles may be used to treat cardiac failure, bone marrow disease, skin disease, burns and degenerative diseases such as diabetes, Alzheimer's disease, Parkinson's disease and cancer.

Such particles may also be used to treat myocardial infarction, a cutaneous wound, a dermatologic disorder, a dermatological lesion, dermatitis, psoriasis, condyloma, verruca, hemangioma, keloid, skin cancer, atopic dermatitis, Behcet disease, chronic granulomatous disease, cutaneous T cell lymphoma, ulceration, a pathological condition characterised by initial injury inducing inflammation and immune dysregulation leading to chronic tissue remodeling including fibrosis and loss of function, renal ischemic injury, cystic fibrosis, sinusitis and rhinitis or an orthopaedic disease.

The particles may be used to aid wound healing, scar reduction, bone formation, a bone graft or bone marrow transplantation in an individual.

Unless the context dictates otherwise, the term "conditioned medium" should be taken to include not only cell culture medium exposed to MSCs as well as such a composition comprising one or more, preferably substantially all, the polypeptides which are present in the conditioned medium.

The particles may also be used as sources for any of the proteins secreted or expressed by the MSCs. We therefore provide for a method of producing a polypeptide as shown in any of Tables D1 to D5, the method comprising obtaining a particle as described, and isolating the polypeptide from the particle.

HEART DISEASE

The mesenchymal stem cell particle methods and compositions described here may be used for treatment or prevention of heart disease.

Heart disease is an umbrella term for a variety for different diseases affecting the heart. As of 2007, it is the leading cause of death in the United States, England, Canada and Wales, killing one person every 34 seconds in the United States alone. Heart disease includes any of the following.

Coronary Heart Disease

Coronary artery disease is a disease of the artery caused by the accumulation of atheromatous plaques within the walls of the arteries that supply the myocardium. Angina pectoris (chest pain) and myocardial infarction (heart attack) are symptoms of and conditions caused by coronary heart disease. Over 459,000 Americans die of coronary heart disease every year. In the United Kingdom, 101,000 deaths annually are due to coronary heart disease.

Cardiomyopathy

Cardiomyopathy is the deterioration of the function of the myocardium (i.e., the actual heart muscle) for any reason. People with cardiomyopathy are often at risk of arrhythmia and/or sudden cardiac death. Extrinsic cardiomyopathies - cardiomyopathies where the primary pathology is outside the myocardium itself comprise the majority of cardiomyopathies . By far the most common cause of a cardiomyopathy is ischemia.

The World Health Organization includes as specific cardiomyopathies: Alcoholic cardiomyopathy, coronary artery disease, congenital heart disease, nutritional diseases affecting the heart, ischemic (or ischaemic) cardiomyopathy, hypertensive cardiomyopathy, valvular cardiomyopathy, inflammatory cardiomyopathy.

Also included are:

Cardiomyopathy secondary to a systemic metabolic disease

Intrinsic cardiomyopathies (weakness in the muscle of the heart that is not due to an identifiable external cause)

Dilated cardiomyopathy (DCM, the most common form, and one of the leading indications for heart transplantation. In DCM the heart (especially the left ventricle) is enlarged and the pumping function is diminished)

Hypertrophic cardiomyopathy (HCM or HOCM, a genetic disorder caused by various mutations in genes encoding sarcomeric proteins. In HCM the heart muscle is thickened, which can obstruct blood flow and prevent the heart from functioning properly),

Arrhythmogenic right ventricular cardiomyopathy (ARVC, which arises from an electrical disturbance of the heart in which heart muscle is replaced by fibrous scar tissue. The right ventricle is generally most affected)

Restrictive cardiomyopathy (RCM, which is the least common cardiomyopathy. The walls of the ventricles are stiff, but may not be thickened, and resist the normal filling of the heart with blood).

Noncompaction Cardiomyopathy - the left ventricle wall has failed to properly grow from birth and such has a spongy appearance when viewed during an echocardiogram.

Cardiovascular Disease

Cardiovascular disease is any of a number of specific diseases that affect the heart itself and/or the blood vessel system, especially the veins and arteries leading to and from the heart. Research on disease dimorphism suggests that women who suffer with cardiovascular disease usually suffer from forms that affect the blood vessels while men usually suffer from forms that affect the heart muscle itself. Known or associated causes of cardiovascular disease include diabetes mellitus, hypertension, hyperhomocysteinemia and hypercholesterolemia.

Types of cardiovascular disease include atherosclerosis

Ischaemic Heart Disease

Ischaemic heart disease is disease of the heart itself, characterized by reduced blood supply to the organs. This occurs when the arteries that supply the oxygen and the nutrients gets stopped and the heart will not get enough of the oxygen and the nutrients and will eventually stop beating.

Heart Failure

Heart failure, also called congestive heart failure (or CHF), and congestive cardiac failure (CCF), is a condition that can result from any structural or functional cardiac disorder that impairs the ability of the heart to fill with or pump a sufficient amount of blood throughout the body. Cor pulmonale is a failure of the right side of the heart.

Hypertensive Heart Disease

Hypertensive heart disease is heart disease caused by high blood pressure, especially localised high blood pressure. Conditions that can be caused by hypertensive heart disease include: left ventricular hypertrophy, coronary heart disease, (Congestive) heart failure, hypertensive cardiomyopathy, cardiac arrhythmias, inflammatory heart disease, etc.

Inflammatory heart disease involves inflammation of the heart muscle and/or the tissue surrounding it. Endocarditis comprises inflammation of the inner layer of the heart, the endocardium. The most common structures involved are the heart valves. Inflammatory cardiomegaly. Myocarditis comprises inflammation of the myocardium, the muscular part of the heart.

Valvular Heart Disease

Valvular heart disease is disease process that affects one or more valves of the heart. The valves in the right side of the heart are the tricuspid valve and the pulmonic valve. The valves in the left side of the heart are the mitral valve and the aortic valve. Included are aortic valve stenosis, mitral valve prolapse and valvular cardiomyopathy.

[The above text is adapted from Heart disease. (2009, February 3). In Wikipedia, The Free Encyclopedia. Retrieved 06:33, February 20, 2009, from http://en.wikipedia.org/w/index.php?title=Heart_disease&oldid=268290924]

DELIVERY OF PARTICLES

The particles as described in this document may be delivered to the human or animal body by any suitable means.

We therefore describe a delivery system for delivering a particles as described in this document to a target cell, tissue, organ, animal body or human body, and methods for using the delivery system to deliver particles to a target.

The delivery system may comprise a source of particles, such as a container containing the particles. The delivery system may comprise a dispenser for dispensing the particles to a target.

Accordingly, we provide a delivery system for delivering a particles, comprising a source of particles as described in this document together with a dispenser operable to deliver the particles to a target.

We further provide for the use of such a delivery system in a method of delivering a particles to a target.

Delivery systems for delivering fluid into the body are known in the art, and include injection, surgical drips, cathethers (including perfusion cathethers) such as those described in United States Patent 6,139,524, for example, drug delivery catheters such as those described in United States Patent 7,122,019.

Delivery to the lungs or nasal passages, including intranasal delivery, may be achieved using for example a nasal spray, puffer, inhaler, etc as known in the art (for example as shown in United States Design Patent D544,957.

Delivery to the kidneys may be achieved using an intra-aortic renal delivery catheter, such as that described in United States Patent 7,241,273.

It will be evident that the particular delivery should be configurable to deliver the required amount of particles at the appropriate interval, in order to achieve optimal treatment.

The particles may for example be used for the treatment or prevention of atherosclerosis. Here, perfusion of particles may be done intravenously to stabilize atherosclerotic plaques or reduce inflammation in the plaques. The particles may be used for the treatment or prevention of septic shock by intravenous perfusion.

The particles may be used for the treatment or prevention of heart failure. This may be achieved by chronic intracoronary or intramyocardially perfusion of particles to retard remodeling or retard heart failure. The particles may be used for the treatment or prevention of lung inflammation by intranasal delivery.

The particles may be used for the treatment or prevention of dermatological conditions e.g. psoriasis. Long term delivery of particles may be employed using transdermal microinjection needles until the condition is resolved.

It will be evident that the delivery method will depend on the particular organ to which the particles is to be delivered, and the skilled person will be able to determine which means to employ accordingly.

As an example, in the treatment of cardiac inflammation, the particles may be delivered for example to the cardiac tissue (i.e., myocardium, pericardium, or endocardium) by direct intracoronary injection through the chest wall or using standard percutaneous catheter based methods under fluoroscopic guidance for direct injection into tissue such as the myocardium or infusion of an inhibitor from a stent or catheter which is inserted into a bodily lumen.

Any variety of coronary catheter, or a perfusion catheter, may be used to administer the compound. Alternatively the particles may be coated or impregnated on a stent that is placed in a coronary vessel.

TISSUE REGENERATION

Mesenchymal stem cells and differentiated cells made according to the methods and compositions described here, and particles derived therefrom, may also be used for tissue reconstitution or regeneration in a human patient in need thereof. The cells are administered in a manner that permits them to graft to the intended tissue site and reconstitute or regenerate the functionally deficient area.

For example, the methods and compositions described here may be used to modulate the differentiation of stem cells. Mesenchymal stem cells and differentiated cells and particles derived therefrom may be used for tissue engineering, such as for the growing of skin grafts. Modulation of stem cell differentiation may be used for the bioengineering of artificial organs or tissues, or for prosthetics, such as stents.

CANCER

Mesenchymal stem cells and differentiated cells made by the methods and compositions described here and particles derived therefrom may be used for the treatment of cancer.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia.

More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastric cancer, pancreatic cancer, glial cell tumors such as glioblastoma and neurofibromatosis, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, renal cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. Further examples are solid tumor cancer including colon cancer, breast cancer, lung cancer and prostrate cancer, hematopoietic malignancies including leukemias and lymphomas, Hodgkin's disease, aplastic anemia, skin cancer and familiar adenomatous polyposis. Further examples include brain neoplasms, colorectal neoplasms, breast neoplasms, cervix neoplasms, eye neoplasms, liver neoplasms, lung neoplasms, pancreatic neoplasms, ovarian neoplasms, prostatic neoplasms, skin neoplasms, testicular neoplasms, neoplasms, bone neoplasms, trophoblastic neoplasms, fallopian tube neoplasms, rectal neoplasms, colonic neoplasms, kidney neoplasms, stomach neoplasms, and parathyroid neoplasms. Breast cancer, prostate cancer, pancreatic cancer, colorectal cancer, lung cancer, malignant melanoma, leukaemia, lympyhoma, ovarian cancer, cervical cancer and biliary tract carcinoma are also included.

The mesenchymal stem cells and differentiated cells made according to the methods and compositions described here may also be used in combination with

anticancer agents such as endostatin and angiostatin or cytotoxic agents or chemotherapeutic agent. For example, drugs such as such as adriamycin, daunomycin, cis-platinum, etoposide, taxol, taxotere and alkaloids, such as vincristine, and antimetabolites such as methotrexate. The term "cytotoxic agent" as used herein refers to a substance that inhibits or prevents the function of cells and/or causes destruction of cells. The term is intended to include radioactive isotopes (e.g. I, Y, Pr), chemotherapeutic agents, and toxins such as enzymatically active toxins of bacterial, fungal, plant or animal origin, or fragments thereof.

Also, the term includes oncogene product/tyrosine kinase inhibitors, such as the bicyclic ansamycins disclosed in WO 94/22867; 1,2-bis(arylamino) benzoic acid derivatives disclosed in EP 600832; 6,7-diamino-phthalazin-1-one derivatives disclosed in EP 600831; 4,5-bis(arylamino)-phthalimide derivatives as disclosed in EP 516598; or peptides which inhibit binding of a tyrosine kinase to a SH2-containing substrate protein (see WO 94/07913, for example). A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer. Examples of chemotherapeutic agents include Adriamycin, Doxorubicin, 5-Fluorouracil (5-FU), Cytosine arabinoside (Ara-C), Cyclophosphamide, Thiotepa, Busulfan, Cytoxin, Taxol, Methotrexate, Cisplatin, Melphalan, Vinblastine, Bleomycin, Etoposide, Ifosfamide, Mitomycin C, Mitoxantrone, Vincristine, VP-16, Vinorelbine, Carboplatin, Teniposide, Daunomycin, Carminomycin, Aminopterin, Dactinomycin, Mitomycins, Nicotinamide, Esperamicins (see U.S. Pat. No. 4,675,187), Melphalan and other related nitrogen mustards, and endocrine therapies (such as diethylstilbestrol (DES), Tamoxifen, LHRH antagonizing drugs, progestins, anti-progestins etc).

OBTAINING MESENCHYMAL STEM CELLS (MSC)

The particles described here may be isolated or produced from mesenchymal stem cell conditioned medium (MSC-CM). MSCs suitable for use in the production of conditioned media and particles may be made by any method known in the art.

In particular, MSCs may be made by propagating a cell obtained by dispersing a embryonic stem (ES) cell colony, or a descendent thereof, in the absence of co-culture in a serum free medium comprising FGF2. This is described in detail in the sections below.

The prior art methods of obtaining mesenchymal stem cells (MSC) or MSC-like cells from hESCs involve either transfection of a human telomerase reverse transcriptase

(hTERT) gene into differentiating hESCs (Xu et al., 2004) or coculture with mouse OP9 cell line (Barberi et al., 2005). The use of exogenous genetic material and mouse cells in these derivation protocols introduces unacceptable risks of tumorigenicity or infection of xenozootic infectious agents.

The particles may therefore be made from MSCs derived by the use of a clinically relevant and reproducible protocol for isolating similar or identical (such as homogenous) MSC populations from differentiating hESCs. In general, the method comprises dispersing a embryonic stem (ES) cell colony into cells. The cells are then plated out and propagated. The cells are propagated in the absence of co-culture in a serum free medium comprising fibroblast growth factor 2 (FGF2), in order to obtain mesenchymal stem cells (MSCs).

Thus, the protocol does not require serum, use of mouse cells or genetic manipulations and requires less manipulations and time, and is therefore highly scalable. The protocol may be used for the isolation of MSCs from two different hESC lines, HuES9 and H-1 and also a third one, Hes-3. Human ES cell derived MSCs (hESC-MSCs) obtained by the methods and compositions described here are remarkably similar to bone-marrow derived MSCs (BM-MSCs).

The embryonic stem cell culture may comprise a human embryonic stem cell (hESC) culture.

In a one embodiment, a method of generating mesenchymal stem cells (MSC) comprises trypsinizing and propagating hESCs without feeder support in media supplemented with FGF2 and optionally PDGF AB before sorting for CD105+CD24- cells.

The method may comprise sorting for CD105+, CD24- cells from trypsinized hESCs one week after feeder-free propagation in a media supplemented with FGF2 and optionally PDGF AB will generate to generate a hESC-MSC cell culture in which at least some, such as substantially all, or all cells are similar or identical (such as homogenous) to each other.

The MSCs produced by this method may be used to produce mesenchymal stem cell conditioned medium (MSC-CM), from which the particles may be isolated.

Disaggregating Embryonic Stem Cell Colonies

One method of producing mesenchymal stem cells may comprise dispersing or disaggregating an embryonic stem cell colony into cells.

The embryonic stem cell colony may comprise a huES9 colony (Cowan CA, Klimanskaya I, McMahon J, Atienza J, Witmyer J, et al. (2004) *Derivation of embryonic stem-cell lines from human blastocysts*. N Engl J Med 350: 1353-1356) or a H1 ESC colony (Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, et al. (1998) *Embryonic Stem Cell Lines Derived from Human Blastocysts*. Science 282: 1145-1147.).

The cells in the colony may be disaggregated or dispersed to a substantial extent, i.e., at least into clumps. The colony may be disaggregated or dispersed to the extent that all the cells in the colony are single, i.e., the colony is completely disaggregated.

The disaggregation may be achieved with a dispersing agent.

The dispersing agent may be anything that is capable of detaching at least some embryonic stem cells in a colony from each other. The dispersing agent may comprise a reagent which disrupts the adhesion between cells in a colony, or between cells and a substrate, or both. The dispersing agent may comprise a protease.

The dispersing agent may comprise trypsin. The treatment with trypsin may last for example for 3 minutes or thereabouts at 37 degrees C. The cells may then be neutralised, centrifuged and resuspended in medium before plating out.

The method may comprise dispersing a confluent plate of human embryonic stem cells with trypsin and plating the cells out.

The disaggregation may comprise at least some of the following sequence of steps: aspiration, rinsing, trypsinization, incubation, dislodging, quenching , re-seeding and aliquoting. The following protocol is adapted from the Hedrick Lab, UC San Diego (http://hedricklab.ucsd.edu/Protocol/COSCell.html).

In the aspiration step, the media is aspirated or generally removed from the vessel, such as a flask. In the rinsing step, the cells are rinsed with a volume, for example 5-10 mls, of a buffered medium, which is may be free from Ca^{2+} and Mg^{2+}. For example, the cells may be rinsed with calcium and magnesium free PBS. In the trypsinization step, an amount of dispersing agent in buffer is added to the vessel, and the vessel rolled to coat the growing surface with the dispersing agent solution. For example, 1 ml of trypsin in Hank's BSS may be added to a flask.

In the incubation step, the cells are left for some time at a maintained temperature. For example, the cells may be left at 37°C for a few minutes (e.g., 2 to 5 minutes). In the

dislodging step, the cells may be dislodged by mechanical action, for example by scraping or by whacking the side of the vessel with a hand. The cells should come off in sheets and slide down the surface.

In the quenching step, a volume of medium is added to the flask. The medium may comprise a neutralising agent to stop the action of the dispersing agent. For example, if the dispersing agent is a protease such as trypsin, the medium may contain a protein, such as a serum protein, which will mop up the activity of the protease. In a particular example, 3 ml of serum containing cell culture medium is added to the flask to make up a total of 4 mls. The cells may be pipetted to dislodge or disperse the cells.

In the re-seeding step, the cells are re-seeded into fresh culture vessels and fresh medium added. A number of re-seedings may be made at different split ratios. For example, the cells may be reseeded at 1/15 dilution and 1/5 dilution. In a particular example, the cells may be re-seeded by adding 1 drop of cells into a $25cm^2$ flask and 3 drops into another to re-seed the culture, and 7-8 mls media is then added to each to provide for 1/15 dilution and 1/5 dilution from for example a $75cm^2$ flask. In the aliquoting step, the cells may be aliquoted into new dishes or whatever split ratio is desired, and media added.

In a specific embodiment, the method includes the following steps: human ES cells are first grown suspended in non-adherent manner to form embryoid bodies (EBs). 5-10 day old EBs are then trypsinized before plating as adherent cells on gelatine coated tissue culture plates.

Maintenance as Cell Culture

The disaggregated cells may be plated and maintained as a cell culture.

The cells may be plated onto a culture vessel or substrate such as a gelatinized plate. Crucially, the cells are grown and propagated without the presence of co-culture, e.g., in the absence of feeder cells.

The cells in the cell culture may be grown in a serum-free medium which is supplemented by one or more growth factors such as fibroblast growth factor 2 (FGF2) and optionally platelet-derived growth factor AB (PDGF AB), at for example 5ng/ml. The cells in the cell culture may be split or subcultured 1:4 when confluent, by treatment with trypsin, washing and replating.

Absence of Co-Culture

The cells may be cultured in the absence of co-culture. The term "co-culture" refers to a mixture of two or more different kinds of cells that are grown together, for example, stromal feeder cells.

Thus, in typical ES cell culture, the inner surface of the culture dish is usually coated with a feeder layer of mouse embryonic skin cells that have been treated so they will not divide. The feeder layer provides an adherent surface to enable the ES cells to attach and grow. In addition, the feeder cells release nutrients into the culture medium which are required for ES cell growth. In the methods and compositions described here, the ES and MSC cells may be cultured in the absence of such co-culture.

The cells may be cultured as a monolayer or in the absence of feeder cells. The embryonic stem cells may be cultured in the absence of feeder cells to establish mesenchymal stem cells (MSC).

The dissociated or disaggregated embryonic stem cells may be plated directly onto a culture substrate. The culture substrate may comprise a tissue culture vessel, such as a Petri dish. The vessel may be pre-treated. The cells may be plated onto, and grow on, a gelatinised tissue culture plate.

An example protocol for the gelatin coating of dishes follows. A solution of 0.1% gelatin in distilled water is made and autoclaved. This may be stored at room temp. The bottom of a tissue culture dish is covered with the gelatin solution and incubated for 5-15min. Remove gelatin and plates are ready to use. Medium should be added before adding cells to prevent hypotonic lysis.

Serum Free Media

The dissociated or disaggregated embryonic stem cells may be cultured in a medium which may comprise a serum-free medium.

The term "serum-free media" may comprise cell culture media which is free of serum proteins, e.g., fetal calf serum. Serum-free media are known in the art, and are described for example in US Patents 5,631,159 and 5,661,034. Serum-free media are commercially available from, for example, Gibco-BRL (Invitrogen).

The serum-free media may be protein free, in that it may lack proteins, hydrolysates, and components of unknown composition. The serum-free media may comprise chemically-defined media in which all components have a known chemical

structure. Chemically-defined serum-free media is advantageous as it provides a completely defined system which eliminates variability allows for improved reproducibility and more consistent performance, and decreases possibility of contamination by adventitious agents.

The serum-free media may comprise Knockout DMEM media (Invitrogen-Gibco, Grand Island, New York).

The serum-free media may be supplemented with one or more components, such as serum replacement media, at a concentration of for example, 5%, 10%, 15%, etc. The serum-free media may comprise or be supplemented with 10% serum replacement media from Invitrogen- Gibco (Grand Island, New York).

Growth Factor

The serum-free medium in which the dissociated or disaggregated embryonic stem cells are cultured may comprise one or more growth factors. A number of growth factors are known in the art, including PDGF, EGF, TGF-a, FGF, NGF, Erythropoietin, TGF-b, IGF-I and IGF-II.

The growth factor may comprise fibroblast growth factor 2 (FGF2). The medium may also contain other growth factors such as platelet-derived growth factor AB (PDGF AB). Both of these growth factors are known in the art. The method may comprise culturing cells in a medium comprising both FGF2 and PDGF AB.

Alternatively, or in addition, the medium may comprise or further comprise epidermal growth factor (EGF). Use of EGF may enhance growth of MSCs. EGF may be used at any suitable concentration, for example 5-10 ng/ml EGF. EGF may be used in place of PDGF. EGF is a protein well known in the art, and is referred to as symbol EGF, Alt. Symbols URG, Entrez 1950, HUGO 3229, OMIM 131530, RefSeq NM_001963, UniProt P01133.

Thus, we disclose the use of media comprising (i) FGF2, (ii) FGF2 and PDGF and (iii) FGF2 and EGF and other combinations.

FGF2 is a wide-spectrum mitogenic, angiogenic, and neurotrophic factor that is expressed at low levels in many tissues and cell types and reaches high concentrations in brain and pituitary. FGF2 has been implicated in a multitude of physiologic and pathologic processes, including limb development, angiogenesis, wound healing, and

tumor growth. FGF2 may be obtained commercially, for example from Invitrogen-Gibco (Grand Island, New York).

Platelet Derived Growth Factor (PDGF) is a potent mitogen for a wide range of cell types including fibroblasts, smooth muscle and connective tissue. PDGF, which is composed of a dimer of two chains termed the A chain and B chain, can be present as AA or BB homodimers or as an AB heterodimer. Human PDGF-AB is a 25.5 kDa homodimer protein consisting of 13.3 kDa A chain and 12.2 B chain. PDGF AB may be obtained commercially, for example from Peprotech (Rocky Hill, New Jersey).

The growth factor(s), such as FGF2 and optionally PDGF AB, may be present in the medium at concentrations of about 100pg/ml, such as about 500pg/ml, such as about 1ng/ml, such as about 2ng/ml, such as about 3ng/ml, such as about 4ng/ml, such as about 5ng/ml. In some embodiments, the medium contains FGF2 at about 5ng/ml. The medium may also contain PDGF AB, such as at about 5ng/ml.

Splitting Cells

Cells in culture will generally continue growing until confluence, when contact inhibition causes cessation of cell division and growth. Such cells may then be dissociated from the substrate or flask, and "split", subcultured or passaged, by dilution into tissue culture medium and replating.

The methods and compositions described here may therefore comprise passaging, or splitting during culture. The cells in the cell culture may be split at a ratio of 1:2 or more, such as 1:3, such as 1:4, 1:5 or more. The term "passage" designates the process consisting in taking an aliquot of a confluent culture of a cell line, in inoculating into fresh medium, and in culturing the line until confluence or saturation is obtained.

Selection, Screening or Sorting Step

The method may further comprise a selection or sorting step, to further isolate or select for mesenchymal stem cells.

The selection or sorting step may comprise selecting mesenchymal stem cells (MSC) from the cell culture by means of one or more surface antigen markers. The use of a selection or sorting step further enhances the stringency of sorting and selection specificity for MSCs and furthermore potentially reduces possible contamination from embryonic stem cells such as hESCs and other hESC-derivatives from the starting

material. This would then further reduce the risk of teratoma formation and further increase the clinical relevance of the protocol we describe.

A number of methods are known for selection or sorting based on antigen expression, and any of these may be used in the selection or sorting step described here. The selection or sorting may be achieved by means of fluorescence activated cell sorting (FACS). Thus, as known in the art, FACS involves exposing cells to a reporter, such as a labelled antibody, which binds to and labels antigens expressed by the cell. Methods of production of antibodies and labelling thereof to form reporters are known in the art, and described for example in Harlow and Lane. The cells are then passed through a FACS machine, which sorts the cells from each other based on the labelling. Alternatively or in addition, magnetic cell sorting (MACS) may be employed to sort the cells.

We have realised that while a number of candidate surface antigens known to be associated with MSCs e.g. CD105, CD73, ANPEP, ITGA4 (CD49d), PDGFRA, some of the MSC associated surface antigens e.g. CD29 and CD49e are also highly expressed in ES cells such as hESCs and their expression are verified by FACS analysis. The association of a surface antigen with MSCs may not be sufficient to qualify the antigen as a selectable marker for isolating MSCs from ES cells such as hESC. Accordingly, the selection or sorting step may employ antigens which are differentially expressed between MSCs and ES cells.

The selection or sorting step of our method may positively select for mesenchymal stem cells based on the expression of antigens. Such antigens may be identified by, for example, comparing the gene expression profiles of hESCs and hESCMSCs. In particular embodiments, the selection or sorting may specifically make use of any of the antigens shown in Table E1A and E1B below.

The selection or sorting step of our method may positively select for mesenchymal stem cells based on the expression of antigens which are identified as expressed on MSCs, but not expressed on ES cells such as hESCs.

CD73 is highly expressed on MSCs, while being not highly expressed on hESCs. Both CD73 and CD105 are highly expressed surface antigens in MSCs and are among the top 20 highly expressed surface antigens in hESC-MSCs relative to hESC, the use of either CD73 or CD105 (or both) as selectable marker for putative MSCs will be equally effective in sorting for putative MSCs generated by differentiating hESCs.

Alternatively, or in addition, the selection or sorting step may negatively select against antigens based on surface antigens that are highly expressed as surface antigen on embryonic stem cells (ES cells) such as hESCs, and not mesenchymal stem cells e.g., hESC-MSC. Selection or sorting may be based on known or previously identified hESC-specific surface antigens such as MIBP, ITGB1BP3 and PODXL, and CD24.

FACS analysis confirms the expression of CD24 on hESC but not hESC-MSCs. Therefore, CD24 may be used as a negative selection or sorting marker either on its own, or in conjunction with CD105 as a positive selectable marker for isolating putative MSCs from differentiating hESC cultures.

EXAMPLES

Mesenchymal stem cells (MSCs) derived from adult bone marrow have emerged as one of the most promising stem cell types for treating cardiovascular disease (Pittenger and Martin, 2004). Although the therapeutic effects of autologous MSCs have been attributed to their potential to differentiate into many different reparative or replacement cell types such as cardiomyocytes, endothelial cells and vascular smooth cells (Minguell and Erices, 2006; Zimmet and Hare, 2005), the differentiation efficiency of transplanted MSCs into therapeutically relevant numbers of functional reparative cells in injured tissues remains to be established.

Recent reports suggest that some of these reparative effects are mediated by paracrine factors secreted by MSCs (Caplan and Dennis, 2006a; Gnecchi et al., 2005; Gnecchi et al., 2006; Schafer and Northoff, 2008). This paracrine hypothesis introduces a radically different dimension to the use of stem cells, particularly MSCs, in regenerative medicine. Potential mechanisms of MSC paracrine actions include endogenous regenerative capacity, angio- and arteriogenesis, attenuating remodeling, and reducing apoptosis. If the therapeutic effects of MSCs are partly mediated by their secretions, the repertoire of stem cell-based therapies could be extended by application of their secreted factors. Such an approach could potentially provide an “off-the-shelf” MSC-based therapeutic option, which is a requisite for time-sensitive protection against reperfusion injury in patients with acute MI, at affordable costs and with excellent quality control and consistency.

In support of this paracrine hypothesis, many studies have identified the presence of cytokines, chemokines and growth factors that could potentially repair injured cardiac

tissues mainly through cardiac and vascular tissue growth and regeneration (Caplan and Dennis, 2006b; Liu and Hwang, 2005). We further supported this hypothesis by performing the first in-depth proteomic analysis of the MSC paracrine secretion (Sze et al., 2007). This was facilitated by our derivation of highly expandable and identical MSC cultures from human ESCs (Lian et al., 2007) and the use of a chemically defined medium to culture the cells and harvest the secretion via the conditioned medium (CM). Surprisingly, many of the secreted proteins were intracellular proteins and are not known to be secreted or transported across plasma membrane. Computational analysis of the secretome predicted that collectively, the secretome has the potential to repair injured tissues such as myocardial ischemia/reperfusion (MI/R) injury (Sze et al., 2007).

To test the computational prediction, the secretion in the form of CM was administered to a pig model of MI/R injury (Timmers et al., 2008). During myocardial ischemia caused by occlusion of a coronary artery, reperfusion therapy is currently the most effective treatment modality. However, reperfusion which involves the opening of the blocked artery to restore blood flow or reperfusion also induces injury to the newly perfused ischemic tissues (Saraste et al., 1997). Therefore the effectiveness of reperfusion therapy could be greatly improved if reperfusion injury could be neutralized immediately at the point of reperfusion. When the CM was delivered intra-coronary to the pig model of MI/R injury immediately after reperfusion, there was a 60% reduction in myocardial infarction, preservation of cardiac function and reduced oxidative stress as early as 4hours after reperfusion. This confirmed that the paracrine secretion of MSC can ameliorate MI/R injury in a clinically relevant animal model (Timmers et al., 2008).

However, the mechanism by which the secretion mediates this immediate effect on MI/R injury is not clear. It is obvious that the immediacy of this protective effect precludes the relatively lengthy process of tissue regeneration as part of the mechanism. Also, many of the secreted proteins are intracellular proteins and are not known to cross plasma membranes readily. To better understand the pararcine effects of MSCs, we systematically fractionated the CM using membranes with different molecular weights cut-off (MWCO) to identify and profile the composition of the active fraction. We previously showed that CM filtered through 0.2 μM membrane but not 1000 kDa MWCO was cardioprotective (Timmers et al., 2008). However, CM partially concentrated against

membrane with1000 kDa MWCO was cardioprotective. This suggested that the cardioprotective effect was mediated by large complexes with diameter of 50 -100 nm.

Here we extended our previously reported list of proteins in the secretion to >700 proteins and these proteins include many proteins that are commonly found in exosmes. We also identified the presence of RNA (<300 nts) in the secretion. Furthermore, the proteins and RNA were encapsulated in phospholipid vesicles and the only detectable particles in the secretion within the hydrodynamic radius (r_h) range of 1-1000 nm were of r_h = 45-55 nm. These particles eluted as a single peak on HPLC fractionation. Together, these studies demonstrate that the large cardioprotective complex in the secretion carry many of the distinctive feature of an exosome leading to our hypothesis that the active cardioprotective component in the secretion is an exosome.

Example 1. Materials and Methods: MSC-CM Preparation

The protocols for MSC generation and CM preparation have been described previously[15, 16].

In short, a chemically defined serum free culture medium is conditioned by MSCs derived from human embryonic stem cells (hESCs), using a clinically compliant protocol. Three polyclonal, karyotypically stable, and phenotypically MSC-like cultures, that do not express pluripotency-associated markers but displayed MSC-like surface antigens (CD29+, CD44+, CD49a+/e+, CD105+, CD166+, CD34-, CD45-) and gene expression profile, are generated by trypsinization and propagation of hESCs from either HuES9 hESC line or H1 hESC line in feeder- and serum-free selection media[15].

One of these cultures, HuES9.E1 can be stably expanded for at least 80 population doublings. To harvest MSC secretion, hESC-derived MSC cultures are transferred to a chemically defined, serum free culture medium to condition the medium for three days before the media containing MSC secretions are collected, clarified by centrifugation, concentrated 25 times using 10 kDa MW cut-off ultrafiltration membranes and sterilized by filtration through a 220 nm filter.

The secretory proteome is analyzed by multidimensional protein identification technology (MuDPIT) and cytokine antibody array analysis, and revealed the presence of 201 unique gene products. Computational analyses disclosed that this CM holds potential cytoprotective properties[16].

Example 2. Materials and Methods: Animals

All experiments are performed in accordance with the "Guide for the Care and Use of Laboratory Pigs" prepared by the Institute of Laboratory Animal Resources and with prior approval by the Animal Experimentation Committee of the Faculty of Medicine, Utrecht University, the Netherlands.

Example 3. Materials and Methods: Study Design

Thirty female Dalland Landrace pigs (60-70 kg; IDDLO, Lelystad, The Netherlands), all pretreated with clopidogrel 75 mg/day for 3 days and amiodarone 400 mg/day for 10 days, are randomly assigned to MSC-CM, non-CM, or saline treatment.

The saline group is added to assess a potential effect of fresh, non-conditioned culture medium. In all pigs, MI is induced by 75 minutes of proximal left circumflex coronary artery (LCxCA) ligation and 4 hours of subsequent reperfusion. An ischemic period of 75 minutes is selected to inflict severe myocardial injury without inducing completely transmural myocardial infarction. The 4 hour reperfusion period is used, because infarct size measurement using TTC staining is most reliable after 3 hours of reperfusion[17]. After longer periods of reperfusion, it becomes more difficult to assess oxidative stress status and apoptotic mechanisms.

Treatment is initiated 5 minutes before the onset of reperfusion by intravenous infusion of MSC-CM (1.0 ml, 2.0 mg protein) non-CM or saline. Immediately following reperfusion, an additional intracoronary bolus MSC-CM (4.0 ml, 8.0 mg protein), non-CM or saline is given. Myocardial infarct size and function are assessed 4 hours after reperfusion.

Example 4. Materials and Methods: MI and Operational Procedure

During the entire operation, ECG, Systemic Arterial Pressure, and capnogram are monitored continuously. Under general anesthesia as described before[18], a median sternotomy is performed and two introduction sheets are inserted in the carotid arteries for a 6 Fr guiding catheter and an 8 Fr conductance catheter (CD Leycom, Zoetermeer, the Netherlands).

The distal tip of a Swan Ganz catheter is placed into the pulmonary artery via the internal jugular vein. Transonic flow probes (Transonic Systems Inc, Ithaca, NY) are placed around the proximal aorta and LCxCA to measure cardiac output and coronary

flow, and a wire is placed around the inferior caval vein to enable functional measurements under varying loading conditions for PV loops.

After functional measurements, 10.000 IU of heparin are administered intravenously and sutures are tightened to occlude the proximal LCxCA. Internal defibrillation with 50 J is used when ventricular fibrillation occurred. After 75 minutes of ischemia, the LCxCA is reopened by release of the suture. Immediately following reperfusion, Nitroglycerine (0.1 mg to prevent no-reflow) is infused through the LCxCA via the guiding catheter, followed by intracoronary treatment with MSC-CM, non-CM or saline. After 4 hours of reperfusion, the final functional measurements are performed and the heart is explanted for infarct size analysis.

Example 5. Materials and Methods: Functional Measurements

Left ventricular (LV) pressure and volume are measured using the conductance catheter method, as described previously[18]. LV pressure and volume signals derived from the conductance catheter are displayed and acquired at a 250-Hz sampling rate with a Leycom CFL-512 (CD Leycom).

Data are acquired during steady state and during temporal caval vein occlusion, all with the ventilator turned off at end expiration. Analysis of the pressure-volume loops is performed with custom software as described previously[19]**.** In addition, short-axis epicardial ultrasound images (Prosound SSD-5000, 5-MHz probe UST-5280-5, Aloka Holding Europe AG, Zug, Switzerland) are obtained at the midpapillary muscle level. Wall thickness (WT) of the infarct area, remote area (septum) and LV internal area (LVia) are measured at end diastole (ED) and end systole (ES). Systolic wall thickening (SWT) is calculated as [(WT(ES)-WT(ED))/WT(ED)] * 100%, fractional area shortening (FAS) as [(LVia(ES)- LVia (ED))/ LVia (ED)] * 100%, and left ventricular ejection fraction (LVEF) as [(EDV-ESV)/EDV] * 100%.

The end-diastolic chamber stiffness is quantified by means of linear regression of the end-diastolic pressure-volume relationship. Echocardiography and PV loops are measured before MI, 1 hour after ischemia and 4 hours after reperfusion. To challenge stunned myocardium, additional measurements are performed during pharmaceutically induced stress by intravenous dobutamine infusion (2.5 μg/kg/min and 5.0 μg/kg/min).

Example 6. Materials and Methods: Infarct Size

Just prior to excision of the heart, the LCxCA (pigs) or LCA (mice) is religated at exactly the same spot as for the induction of the MI. Evans blue dye is infused through the coronary system to delineate the area at risk (AAR).

The heart is then excised, the LV is isolated and cut into 5 slices from apex to base. The slices are incubated in 1% triphenyltetrazolium chloride (TTC, Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands) in 37°C Sörensen buffer (13.6 g/L KH_2PO_4 + 17.8 g/L $Na_2H\ PO_4 \cdot 2H_2O$, pH 7.4) for 15 minutes to discriminate infarct tissue from viable myocardium.

All slices are scanned from both sides, and in each slide, the infarct area is compared with area at risk and the total area by use of digital planimetry software (Image J). After correction for the weight of the slices, infarct size is calculated as a percentage of the AAR and of the LV.

Example 7. Materials and Methods: Oxidation induced cell death assay

Human leukemic CEM cells are incubated in either CM or non-CM, and treated with 50 μM H_2O_2 to induce oxidative stress. Cell viability is assessed using Trypan Blue exclusion at 12, 24, 36 and 48 hours after H_2O_2 treatment.

Example 8. Materials and Methods: Immunostaining

Nuclear oxidative stress in the ischemia and reperfusion area is assessed by immunostaining for 8-hydroxy-2'-deoxyguanosine (8-OHdG), a product of oxidative stress to DNA. Tissue samples are fixed in 4% formalin before being embedded in paraffin.

Following antigen retrieval in 10 mM citric acid, the tissue sections are incubated with 10% normal horse serum for 30 minutes, mouse-anti-8-OHdG (OXIS international, Foster City, CA, USA) 1:20 in 0.1% PBSA over night at 4ºC, biotin labeled horse-anti-mouse (Vector laboratories, Burlingame, CA, USA) 1:500 for 1 hour and with streptavidin-HRPO 1:1000 for 1 hour.

Finally, the sections are incubated with H_2O_2-diaminobenzidine for 10 minutes. The amount of 8-OHdG positive nuclei is quantified in 4 randomly picked fields per section with digital image microscopy software Analysis (Olympus, Münster, Germany) at 200x magnification.

Example 9. Materials and Methods: Western Blotting

Protein is isolated from frozen tissue samples collected from the ischemia/reperfusion area of pigs using 1 ml Tripure Isolation Reagent (Boehringer, Mannheim, Germany) according to the manufacturer's protocol. For western blotting, 8 µg total protein is separated on a 10% SDS-PAGE gel, transferred onto a Nitrocellulose C membrane (Amersham, Buckinghamshire, UK) and blocked using Phosphate Buffered Saline (PBS)–0.1% Tween–5% Protifar (Nutricia, Netherlands).

The membrane is incubated with a rabbit antibody for phosphoSMAD2 1:1000 (Cell Signalling Technology), for active caspase 3 1:100 (Chemicon, Germany), or for beta-tubulin 1:5000 (Abcam, Cambridge, UK), and subsequently with goat-anti-rabbit HRP 1:2000 (DAKO, Glostrup, Denmark). Chemiluminescence substrate (NENk Life Science Products) is used for detection; the bands are analyzed using the Gel Doc 1000 system (Biorad, Veenendaal, Netherlands).

Example 10. Materials and Methods: MSC-CM Fractionation

The MSC-CM is prepared by sterile filtration through a 220 nm filter and concentrated through a 10 nm filter and therefore contains components between 10 and 220 nm. Subsequently, a <1000 kDa fraction is prepared by filtering the MSC-CM through a 1000 kDa MW cut-off membrane with a 100 nm nominal pore size (Pall Corporation, Singapore), generating a fraction containing products between 10 and 100 nm.

To identify the fraction containing the factor(s) within the medium that confers cardioprotection (10-100 nm or 100-220 nm), a mouse or pig model of ischemia and reperfusion injury is used. MI is induced by 30 minutes left coronary artery (LCA) occlusion and subsequent reperfusion. Mice are treated with 20 µl unfractionated MSC-CM (10-220 nm), the < 1000 kDa fraction (10-100 nm), or saline intravenously via the tail vein, 5 minutes before reperfusion. Infarct size is assessed 24 hours later using Evans blue and TTC as described previously.

Example 11. Materials and Methods: Data analysis

Data are presented as mean ± SEM. Values are collected in a blinded fashion and compared using one-way ANOVA with post hoc bonferroni tests in SPSS 11.5. P-value < 0.05 is considered significant.

Example 12. Results: Mortality

Four pigs died due to refractory ventricular fibrillation during the ischemia, before treatment, and are therefore excluded from the study. All pigs that are treated with CM (n=9), non-CM (n=9) or saline (n=8) also survived the follow up period.

Example 13. Results: Infarction Size

Infarct size, compared to the area at risk (AAR) as well as compared to the LV, is markedly reduced in pigs treated with MSC-CM compared to those treated with non-CM and saline (Figure 1). MSC-CM treatment resulted in approximately 60% reduction of infarct size. Importantly, the AAR is similar in all pigs, which indicates that the initial ischemic injury is similar in all pigs (Table E1 below).

	baseline			**myocardial infarction**		
parameter	**non-CM**	**CM**	**Saline**	**non-CM**	**CM**	**Saline**
AAR	-	-	-	35.1 ± 2.9	30.7 ± 1.6	32.4 ± 2.3
IS (% of AAR)	-	-	-	62.2 ± 5.0	25.4 ± 4.8 †‡	62.5 ± 8.4
IS (% of LV)	-	-	-	22.3 ± 2.9	7.6 ± 1.4 †‡	20.5 ± 3.1
HR, bpm	83.5 ± 5.5	76.4 ± 5.7	77.6 ± 8.1	111.6 ± 6.3 *	86 ± 7.5 †	97 ± 5.5
MAP, mmHg	93.7 ± 10.2	94.0 ± 5.4	100.9 ± 4.9	70.8 ± 9.1 *	95.8 ± 4.3 ‡	68.5 ± 7.3 *
QLCx, ml/min	31.9 ± 2.8	28.7 ± 2.9	28.4 ± 4.1	29.9 ± 2.6	25.1 ± 2.6	25.6 ± 3.3
CO, l/min	3.60 ± 0.14	3.25 ± 0.17	3.79 ± 0.23	2.61 ± 0.35 *	3.15 ± 0.27 ‡	2.04 ± 0.20 *
WT infarct, cm	0.71 ± 0.04	0.64 ± 0.04	0.71 ± 0.03	1.23 ± 0.09 *	0.93 ± 0.09 *‡	1.30 ± 0.09 *
SWT infarct, %	56.3 ± 5.6	56.9 ± 4.8	61.8 ± 4.3	-6.1 ± 1.4 *	18.7 ± 4.1 *†‡	-7.0 ± 1.5 *
SWT remote, %	43.1 ± 3.3	43.0 ± 3.0	40.9 ± 4.2	43.7 ± 7.4	41.4 ± 6.1	37.8 ± 4.4
FAS, %	42.7 ± 2.9	38.8 ± 2.9	41.3 ± 2.7	24.7 ± 2.6 *	36.5 ± 2.0 †‡	21.7 ± 1.2 *
EDV, ml	90.3 ± 8.2	86.2 ± 9.1	101.5 ± 7.1	70.2 ± 8.3	69.7 ± 5.2 *	69.1 ± 11.6
ESV, ml	47.8 ± 8.2	43.9 ± 6.2	53.0 ± 4.7	44.2 ± 6.0	31.4 ± 1.4 *	47.5 ± 9.6
SV, ml	44.5 ± 3.2	43.3 ± 5.0	49.6 ± 3.6	24.5 ± 4.0 *	38.5 ± 4.2 †‡	21.9 ± 2.7 *
EF, %	52.5 ± 5.0	51.7 ± 3.1	49.0 ± 2.7	38.8 ± 3.5 *	54.2 ± 2.6 †‡	34.7 ± 4.4 *
dP/dtmax, mmHg/s	1290 ± 118	1093 ± 83	1299 ± 76	1592 ± 226	1528 ± 130	1075 ± 68 *

dP/dtmin, mmHg/s	-1614 ± 143	-1329 ± 113	-1650 ± 89	-983 ± 130 *	-1031 ± 116 *	-910 ± 102 *
Stiffness, mmHg/ml	0.13 ± 0.03	0.15 ± 0.03	0.14 ± 0.01	0.29 ± 0.06 *	0.13 ± 0.01 †‡	0.29 ± 0.05 *

Table E1. Hemodynamic and Functional Parameters. Baseline values and myocardial infarction values of pigs treated with non-CM, CM or saline, determined with echocardiography and conductance catheters based LV pressure and volume measurements. AAR indicates area at risk; IS, infarct size; LV, left ventricle; HR, heart rate; QLCx, left circumflex coronary artery flow; CO, cardiac output; WT, wall thickness; SWT, systolic wall thickening; FAS, fractional area shortening; EDV, end-diastolic volume; ESV, end-systolic volume; SV, stroke volume; EF, ejection fraction; Ees, end-systolic elastance. Non-CM, n=9; CM, n=9; saline, n=8. * $p<0.05$ vs. baseline; † $p<0.05$ vs. non-CM; ‡ $p<0.05$ vs. saline.

Example 14. Results: Cardiac Function

Baseline parameters are similar in all groups (Table E1 above). During ischemia, the posterolateral wall became completely dyskinetic in all groups, as is observed by negative values of echocardiographic systolic wall thickening (SWT, Figure 2A). Four hours after reperfusion, the reperfused posterolateral wall of both non-CM and saline control groups, is still dyskinetic. In the pigs treated with MCS-CM, however, SWT partly recovered (Figure 2A).

Intravenous infusion of the β_1-adrenergic receptor agonist dobutamine further increased systolic wall thickening in the MCS-CM treated pigs, whereas no improvement is seen in the control groups. Also global left ventricular systolic function decreased due to the ischemia (Figure 2B). In the pigs treated with CM, the fractional area shortening increased after reperfusion, almost back to the baseline level, and increased above baseline level during dobutamine infusion.

In control pigs, global systolic function remained impaired. Improved cardiac function also became evident from the PV-loop derived indices (Table E1). Left ventricular EF and stroke volume are significantly higher in CM treated pigs. This translated into improved hemodynamic parameters such as cardiac output, mean arterial pressure and heart rate.

Diastolic function decreased in control groups following ischemia and reperfusion injury, as observed by increased end-diastolic myocardial stiffness. In the CM treated pigs however diastolic function is not impaired.

Example 15. Results: Oxidative Stress

Having demonstrated the reduction in infarct size and improvement of function, we used an *in vitro* assay of hydrogen peroxide (H_2O_2)-induced cell death to determine the effects of CM and non-CM on oxidative stress, a major cause of ischemia reperfusion injury.

We induced hydrogen peroxide (H_2O_2)-mediated oxidative stress in human leukemic CEM cells in the presence of either CM or non-CM and monitored cell viability by Trypan blue-exclusion. Results showed that CM significantly protected against (H_2O_2)-induced loss of cell viability compared to non-CM ($p<0.05$) (Figure 3A).

To determine if CM also reduces oxidative stress in the hearts of CM-treated pigs, nuclear oxidative stress in tissue sections of pigs treated with CM, non-CM or saline is quantified by 8-OHdG immunostaining for oxidized DNA. Intense nuclear staining indicative of DNA oxidation is observed in sections of non-CM or saline-treated pigs compared to CM-treated pigs (Figures 3B-D). In addition, there are also significantly more positive nuclei in non-CM or saline- treated pigs (Figure 3E).

Therefore, CM can confer cytoprotection against oxidative stress *in vitro* and *in vivo*.

Example 16. Results: TGF-β signaling

The secretions of the MSCs contained many proteins involved in TGF-β signaling[16]. To assess the influence of CM treatment on TGF-β signaling *in vivo*, we quantified phosphorylated SMAD-2 in the myocardial tissue samples of CM treated pigs and control pigs by Western blotting. CM treatment resulted in reduced pSMAD2 expression, indicating that TGF-β signaling via ALK-5 is reduced (Figures 4A, B).

Example 17. Results: Apoptosis

Reperfusion injury causes cell death through apoptosis rather than necrosis[20-22]. To verify that CM treatment reduces apoptosis during reperfusion, we quantified the level of active caspase 3, a key mediator of apoptosis, by western blotting. In the pigs treated with MSC-CM, active caspase-3 levels are lower compared to both non-CM control and saline control suggesting that CM inhibits apoptosis *in vivo* (Figures 4C, D).

Example 18. Results: MSC-CM Fractionation

Unfractionated MSC-CM contains products between 10 and 220 nm. In order to come closer to identifying the cardioprotective factor(s) within the MSC-CM, a <1000 kDa fraction is generated containing products ranging in size from 10-100 nm. Unfractionated MSC-CM confers cardioprotection, whereas the <1000 kDa fraction does not (Figure 5), indicating that the cardioprotective factor(s) are in the >1000 kDa fraction with size ranging from 100 to 220 nm.

Example 19. Results: Size Fractionation Did Not Segregate Secreted Proteins According to Molecular Weights

To identify the active component in the Conditioned Medium, we attempted to size-fractionate the Conditioned Medium into distinct MW fractions by filtering the Conditioned Medium through membranes with different MW cut-offs.

When the Conditioned Medium is filtered through a membrane of MW cut-off of 100 kD to generate a retentate to filtrate volume ratio of 4:1, most proteins <100 kD segregated into the >100 kD fraction, and not into the expected <100 kD fraction (Figure 6). The ratio of the individual protein bands in both unfractionated Conditioned Medium and the >100 kD fraction are similar.

Most proteins with MW <300 kD also did not filter through membrane with a MW cut-off of 300 kD (Figure 6). The MW sizes of some of the major protein bands in the filtrate are similar to that in the non-conditioned medium (NCM), and to exogenously added protein supplements in the culture media: insulin-transferrin-selenoprotein supplement (ITS), FGF2, EGF and PDGF AB (Figure 7).

Together these observations suggest that proteins secreted by the cells are in complexes, and these secretion complexes are larger than 100 kD Proteins that are added exogenously to the culture medium as supplements, and that are less than 100 kD are readily filtered through membranes with MW cut off of 100 kD.

Example 20. Results: Biological Activity in Size-Fractionated Conditioned Medium with MW >1,000kD or diameter of 50-150 nm

To determine the upper and lower size limits of the putative secretion complex, we performed size fractionation of the Conditioned Medium and tested the biological acitivity in a mouse of ischemia reperfusion injury. As the Conditioned Medium is filtered through 0.2 μM filter and concentrated against a membrane with a MW cut-off of 10 kD, this

effectively placed the putative secretion complex in the size range of between 2 to 200 nm (Figure 8).

To narrow this size range further, we determine if there are biological activity in the filtrate from filtering the Conditioned Medium completely through membrane with a MW cut-off of 100 kD or 1000kD, retentate from filtering the Conditioned Medium through membrane with a MW cut-off of 1000 kD. The volume of the retentate is 1/5 of the input volume. The fractions are tested on a mouse or pig model of myocardial ischemia (MI) and reperfusion injury.

In this model, MI is induced by 30 minutes left coronary artery (LCA) occlusion by suture ligation and reperfusion is initiated by removal of suture. Mice are treated with 20 µl unfractionated MSC-CM (10-220 nm), 20 µl of < 100 or 1,000 kD fraction, 4 µl of >1000 kD retentate or saline intravenously via the tail vein, 5 minutes before reperfusion. 24 hours later, the hearts are excised. Before excision, the Area At Risk (AAR) is determined by religating the LCA and then perfusing Evans blue through the aorta. AAR is defined as the area not stained by the dye and is expressed as a percentage of the left ventricular wall area. Infarct size is assessed 24 hours later using Evans blue and TTC as described previously.

The relative AAR in all animals are not significantly different (Figure 9). However the relative infarct size is significantly reduced in animals treated with Conditioned Medium and the >1000 kD fraction when compared to saline ($p=0.01$ and 0.05, respectively) (Figure 10). The <100 and <1000 kD fractions are not biologically active, suggesting that the putative active complex is >1000 kD. It is, however still possible that the complex is <1000 kD, and that passing the Conditioned Medium through the filters inactivates the complex

Example 21. Results: Electron Microscopy of Conditioned Medium Revealed the Presence of 50 to 200 nm Particles

Electron microscopy analysis of the Conditioned Medium is performed using standard methodology. Briefly, the Conditioned Medium in PBS is loaded onto formwar carbon coated grids (Ted Pella Inc, Redding, CA, USA cat no 01800N-F), fixed in 2.5% glutaraldehyde, washed, contrasted in 2% uranyl acetate, embedded in a mixture of uranyl acetate (0.8%) and methyl cellulose (0.13%), and examined under an electron microscope. Consistent with the size fractionation studies above, we observed the presence of

numerous vesicles of ~50- 150 nm, suggesting that these vesicles are the putative active complexes in the secretion (Figure 11).

The hypothesis is supported by the observation that when the Conditioned Medium is ultracentrifuged of 200,000×g for an hour, the pellet when assayed by LC/LC-MS as previously described [20] contained at least 70% of the proteins found in the secretion.

Example 22. Results: Lipid Composition of Conditioned Medium

To analyze the lipid composition of the Conditioned Medium, the hydrophobic lipid/steroid components of Conditioned Medium and NCM are extracted by Folch procedure. Briefly, 50 ml of Conditioned Medium or NCM is vigorously mixed with 5ml of chloroform and 2ml of methanol. The organic and aqueous phases are allowed to separate. The bottom chloroform layer is removed and evaporated to dryness by speedvac.

The residue is reconstituted in methanol for LC-MS/MS analysis. The sample is then injected to a normal-phase (silica phase) HPLC column with dichloromethane/methanol/water/ethylamine mobile phase. The eluate is then ionized online by nanospray to LTQ-FTMS/Orbitrap. The LTQ-FTMS/Orbitrap is run at alternate positive and negative modes for detection of lipid/steroid with different chemical properties. The top 5 precursor ions of each MS scan are further analyzed by MS/MS scan

The molecules are therefore characterized by a combination of FTMS and LTQ. The precursor mass of each tandem mass spectrum is first matched to a candidate in a lipid and metabolic database. MS/MS spectra of ions that have a <5 pmm mass error to any molecule in the database are then compared to known standard spectra or spectra predicted by Mass Frontier program

Mass spectrometry analysis of chloroform extract from the Conditioned Medium revealed the presence of lipids commonly found in plasma membrane, namely phospholipids, glycolipids, and steroids and also in exosomes[35]. The phospholipids include phosphatidyl serine and phosphatidyl inositol, phosphatidyl choline, shingomyelin, ceramides; glycolipid such as cerebroside and steroids such as cholesterol.

It has been observed that exosomes has microdomains known as lipid rafts in their lipid membranes[22, 24-35 41, 42] . Exosomes are cholesterol-rich and their cholesterol-phospholipid ratio generally exceed the ratio of 0.3-0.4 (mol/mol) ratio found in plasma membrane[35]. These rafts are characterized by their resistance to dissolution by non-ionic detergents such as Triton X-100 or Brij-98 at low temperatures, and their sensitivity to

cyclodextrin that binds cholesterol. Generally insoluble in detergents such as triton X-100 and detergent insolubility is often used to identify the presence of lipid rafts.

When the Conditioned Medium is treated with Triton X-100, the secreted proteins continued to segregate as a complex independent of their size fractionation experiments using membrane filtration (Figure 12), suggesting that the putative complex is resistant to dissolution by triton X-100, consistent with the presence of lipid rafts [43].

We determine if the complex is sensitive to dissolution in the presence of 20 mM cyclodextrin. If the putative complex has lipid rafts in the membrane, extraction of cholesterol by cyclodextrin caused dissolution and release the proteins which can then be size fractionated according to their molecular sizes. The relative quantitative composition of lipids in the putative complex is estimated using chromatographic and mass spectrometry techniques as previously outlined [35]. This determines if the lipid composition can support the presence of lipid rafts.

Example 23. Results: RNA Composition of Conditioned Medium

Trizol extraction of the Conditioned Medium followed by isopropanol precipitation as commonly used in the extraction of RNA from cells produces a pellet that in water has an 260:280 nm absorbance ratio of 1.9, suggesting that it may be RNA.

This is consistent with a previous report that exosomes contain mRNAs and microRNAs[36]. This pellet is assayed for sensitivity to RNase activity. The Conditioned Medium will also treated with RNases before extraction with trizol. These assays determine if the pellet is RNA, and if the RNA is sequestered in lipid vesicles such as exosomes.

If so, the RNA is assayed by generic gene expression assays such as microarrary, sequencing, RT-PCR and in vitro translation assays to determine the composition and functions of RNA. The RNAs are translated *in vitro* using standard commercially available reticulocyte lysate system with and without ^{15}N-leucine. The translated protein products are identified by mass spectrometry.

Example 24. Results: Proteomic Profile

Of the ~700 proteins described to be present in the secretion (US Provisional Patent Application No. 60/878,222 and International Application PCT/SG2006/000232. Mesenchymal Stem Cell Conditioned Medium), there are many proteins found to be commonly present in the proteome of other exosomes (Figure 13)[44]. There are also many

proteins in the list of ~700 proteins that have not been described to be present in exosomes. Some notable but not exhaustive examples are Thy1, Wnt 5a, Wnt 5b, inhibin A (or activin A)

Example 25. Results: Surface Antigen Profile

The proteomic profile of the Conditioned Medium also describes the presence of proteins that are known to be membrane-bound. Some notable but not exhaustive examples include CD9, CD109, thy-1[20]. Other known surface antigen of exosome such as CD24 that is found on the surface of exosomes secreted in the urine[45] is not expressed in MSCs[19] or its secretion[20].

Additionally, many of these surface antigens are expressed in a cell-type specific manner. Together, these observations suggest that the surface antigen profile will define and distinguish exosomes from different cell source. To characterize the surface antigen profile of these putative secretion complex, the Conditioned Medium is biotinylated using standard commercially available biotinylation kits. The proteins is separated on standard SDS-PAGE, transferred on nylon or nitrocellulose, and probed with avidin-peroxidase using standard protocols in western blot analysis. In this protocol, only proteins that are on the surfaces on the complexes and are physically accessible to biotin are biotinlyated. All the proteins that are within the complex and are therefore not physically accessible are be biotinylated. The biotinylated proteins are also isolated using avidin-affinity chromatography and identify using LC/MS. The identity of these proteins is confirmed by western blot analysis, immunoelectron microscopy and gene expression of MSCs.

Example 26. Exosomes

Based on the above observations, we hypothesize that the smallest active cardioprotective unit in the Conditioned Medium is an exosome.

To prove this hypothesis, we concentrate the Conditioned Medium using membrane filtration technology with membrane of MW cut-off of 100 kD. The concentrated Conditioned Medium is then utlracentrifuged at ~150-200 000 g for 1-2 hours. The pellet is resuspended in PBS, analysed by electron microscopy to confirm the presence of particles with a size range of 50-150 nm, and assayed for its protein, lipid and RNA contents.

The suspension is assayed for the biological activities that are computationally predicted for the Conditioned Medium[20], and is tested for cardioprotective effects in the mouse and pig models as described above or below, respectively.

Example 27. Study Design for Pig Study

Thirty female Dalland Landrace pigs (60-70 kg; IDDLO, Lelystad, The Netherlands), all pretreated with clopidogrel 75 mg/day for 3 days and amiodarone 400 mg/day for 10 days, are randomly assigned to MSC-CM, non-CM, or saline treatment.

The saline group is added to assess a potential effect of fresh, non-conditioned culture medium. In all pigs, MI is induced by 75 minutes of proximal left circumflex coronary artery (LCxCA) ligation and 4 hours of subsequent reperfusion. An ischemic period of 75 minutes is selected to inflict severe myocardial injury without inducing completely transmural myocardial infarction. The 4 hour reperfusion period is used, because infarct size measurement using TTC staining is most reliable after 3 hours of reperfusion[46].

After longer periods of reperfusion, it becomes more difficult to assess oxidative stress status and apoptotic mechanisms. Treatment is initiated 5 minutes before the onset of reperfusion by intravenous infusion of MSC-CM (1.0 ml, 2.0 mg protein) non-CM or saline. Immediately following reperfusion, an additional intracoronary bolus MSC-CM (4.0 ml, 8.0 mg protein), non-CM or saline is given. Myocardial infarct size and function are assessed 4 hours after reperfusion.

To identify the factor(s) within the medium that confer cardioprotection, we used a mouse or pig model of ischemia and reperfusion injury. MI is induced by 30 minutes left coronary artery (LCA) occlusion and subsequent reperfusion. Mice are treated with unfractionated conditioned medium, <1000 kD fraction, <500 kD fraction, <300 fraction kD, <100 kD fraction or saline intravenously via the tail vein, 5 minutes before reperfusion. Infarct size is assessed the following day (24 hours after reperfusion).

Example 28. MI and Operational Procedure

During the entire operation, ECG, Systemic Arterial Pressure, and capnogram are monitored continuously. Under general anesthesia as described before[47], a median sternotomy is performed and two introduction sheets are inserted in the carotid arteries for a 6 Fr guiding catheter and an 8 Fr conductance catheter (CD Leycom, Zoetermeer, the Netherlands).

The distal tip of a Swan Ganz catheter is placed into the pulmonary artery via the internal jugular vein. Transonic flow probes (Transonic Systems Inc, Ithaca, NY) are placed around the proximal aorta and LCxCA to measure cardiac output and coronary flow, and a wire is placed around the inferior caval vein to enable functional measurements under varying loading conditions for PV loops. After functional measurements, 10.000 IU of heparin are administered intravenously and sutures are tightened to occlude the proximal LCxCA. Internal defibrillation with 50 J is used when ventricular fibrillation occurred. After 75 minutes of ischemia, the LCxCA is reopened by release of the suture. Immediately following reperfusion, Nitroglycerine (0.1 mg to prevent no-reflow) is infused through the LCxCA via the guiding catheter, followed by intracoronary treatment with MSC-CM, non-CM or saline. After 4 hours of reperfusion, the final functional measurements are performed and the heart is explanted for infarct size analysis.

Mice are anesthetized with Fentanyl (0.05 mg/kg), Dormicum (5 mg/kg) and Domitor (0.5 mg/kg) and intubated using a 24-gauge intravenous catheter with a blunt end. Mice are artificially ventilated at a rate of 105 strokes/min using a rodent ventilator with a mixture of O_2 and N_2O (1:2 vol/vol) to which isoflurane (2.5–3.0% vol/vol) is added. The mouse is placed on a heating pad to maintain the body temperature at 37°C. The chest is opened in the third intercostal space and an 8-0 prolene suture is used to occlude the left coronary artery (LCA) for 30 minutes. The chest is closed and the following day (24 hours later), the hearts are explanted for infarct size analysis.

Example 29. Functional Measurements

The ECG, arterial pressure and cardiac output, are digitized at a sampling rate of 250 Hz and stored for offline analysis (Leycom CFL-512, CD Leycom). Left ventricular (LV) pressure and volume are measured using the conductance catheter method, as described previously[47]. LV pressure and volume signals derived from the conductance catheter are displayed and acquired at a 250-Hz sampling rate with a Leycom CFL-512 (CD Leycom).

Data are acquired during steady state and during temporal caval vein occlusion, all with the ventilator turned off at end expiration. Analysis of the pressure-volume loops is performed with custom software as described previously[48]**.** In addition, short-axis epicardial ultrasound images (Prosound SSD-5000, 5-MHz probe UST-5280-5, Aloka

Holding Europe AG, Zug, Switzerland) are obtained at the midpapillary muscle level. Wall thickness (WT) of the infarct area, remote area (septum) and LV internal area (LVia) are measured at end diastole (ED) and end systole (ES).

Systolic wall thickening (SWT) is calculated as [(WT(ES)-WT(ED))/WT(ED)] * 100%, fractional area shortening (FAS) as [(LVia(ES)- LVia (ED))/ LVia (ED)] * 100%, and left ventricular ejection fraction (LVEF) as [(EDV-ESV)/EDV] * 100%. The end-diastolic chamber stiffness is quantified by means of linear regression of the end-diastolic pressure-volume relationship. Echocardiography and PV loops are measured before MI, 1 hour after ischemia and 4 hours after reperfusion. To challenge stunned myocardium, additional measurements are performed during pharmaceutically induced stress by intravenous dobutamine infusion (2.5 μg/kg/min and 5.0 μg/kg/min).

Example 30. Infarct Size

Just prior to excision of the heart, the LCxCA (pigs) or LCA (mice) is religated at exactly the same spot as for the induction of the MI. Evans blue dye is infused through the coronary system to delineate the area at risk (AAR). The heart is then excised, the LV is isolated and cut into 5 slices from apex to base.

The slices are incubated in 1% triphenyltetrazolium chloride (TTC, Sigma-Aldrich Chemicals, Zwijndrecht, the Netherlands) in 37°C Sörensen buffer (13.6 g/L KH_2PO_4 + 17.8 g/L $Na_2H\ PO_4 \cdot 2H_2O$, pH 7.4) for 15 minutes to discriminate infarct tissue from viable myocardium.

All slices are scanned from both sides, and in each slide, the infarct area is compared with area at risk and the total area by use of digital planimetry software (Image J). After correction for the weight of the slices, infarct size is calculated as a percentage of the AAR and of the LV.

Example 31. Materials and Methods: Preparation of Conditioned Medium

HuES9.E1 cells are cultured as described previously (Lian et al., 2007; Sze et al., 2007).

Briefly, 80% confluent HuES9.E1 cell cultures are washed three times with PBS and cultured overnight in a chemically defined medium consisting of DMEM without phenol red (catalog number 31053, Invitrogen) and supplemented with insulin, transferrin, and selenoprotein (ITS) (Invitrogen), 5 ng/ml FGF2 (Invitrogen), 5 ng/ml PDGF AB (Peprotech, Rocky Hill, NJ), glutamine-penicillin-streptomycin, and b-mercaptoethanol.

The cultures are then rinsed three times with PBS, and then fresh defined medium is added.

After 3 days, the medium is collected, centrifuged at 500x*g* and concentrated. >100kDa CM sample is prepared by concentrating CM 50x using 100kDa MWCO tangential force filtration (TFF). All other concentrations are performed using ultrafiltration membrane. All CM and other differently processed CMs are 0.2 micron filtered after all procedures and before being stored or used.

Example 32. Materials and Methods: LC MS/MS Analysis

Proteins in two ml of dialyzed conditioned (CM) or non-conditioned media (NCM) are reduced, alkylated, and tryptic digested as described previously described (Sze et al., 2007). The samples are then desalted by passing the digested mixture through a conditioned Sep-Pak C-18 SPE cartridge (Waters, Milford, MA, USA), washed twice with a 3% acetonitrile (ACN) (JT Baker, Phillipsburg, NJ) and 0.1% formic acid (FA) buffer, and eluted with a 70% ACN and 0.1% FA buffer. The eluted samples are then dried to about 10% of their initial volumes by removing organic solvent in a vacuum centrifuge.

To reduce the sample complexity, offline peptide fractionation is carried out with a HPLC system (Shimadzu, Japan) through a Polysulfoethyl SCX column (200mm x 4.6mm) (PolyLC, USA). Mobile phase A (5mM KH4PO4 + 30% acetonitrile) and mobile phase B (5mM KH4PO4 + 30% acetonitrile + 350mM KCl) at 1ml/min. Eight fractions are collected and dried with a vacuum centrifuge. Fractionated samples are loaded into the autosampler of a Shimadzu DGU-20A3 C18 reverse phase HPLC system coupled online to a LTQ-FT ultra linear ion trap mass spectrometer (Thermo Electron, San Jose, CA) fitted with a nano-spray source. Injected peptides are trapped in a Zorvax 300SB-C18 enrichment column (5mm x03mm, Agilent Technologies, Germany) and eluted into a nano-bored C18 packed column (75µm x 100Å, Michrom Bioresources, Auburn, CA).

A 90 minute gradient at 200nl/min flow rate is used to elute the peptides into the mass spectrometer. The LTQ is operated in a data-dependent mode by performing MS/MS scans for the 8 of the most intense peaks from each MS scan in the FTMS. For each experiment, MS/MS (dta) spectra of the eight SCX fractions are combined into a single mascot generic file by a home-written program. Protein identification is achieved by searching the combined data against the IPI human protein database (version 3.34; 67,758 sequences) via an in-house Mascot server (Version 2.2, Matrix Science, UK). The search

parameters are: a maximum of 2 missed cleavages using trypsin; fixed modification is carbaminomethylation of cysteine and variable modifications is oxidation of methionine. The mass tolerances are set to 20 ppm and 0.8 Da for peptide precursor and fragment ions respectively. Protein identifications are accepted as true positive if two different peptides are found to be with scores greater than the homology scores.

Example 33. Materials and Methods: HPLC Fractionation and Dynamic Light Scattering using a Quasi-Elastic Light Scattering (QELS) Detector

The instrument setup consisted of a liquid chromatography system with a binary pump, an auto injector, a thermostated column oven and a UV-visible detector operated by the Class VP software from Shimadzu Corporation (Kyoto, Japan). The Chromatography columns used are TSK Guard column SWXL, 6 x 40 mm and TSK gel G4000 SWXL, 7.8 x 300 mm from Tosoh Corporation (Tokyo, Japan).The following detectors, Dawn 8 (light scattering), Optilab (refractive index) and QELS (dynamic light scattering) are connected in series following the UV-visible detector. The last three detectors are from Wyatt Technology Corporation (California, USA) and are operated by the ASTRA software.

The components of the sample are separated by size exclusion i.e. the larger molecules will elute before the smaller molecules. The eluent buffer used is 20 mM phosphate buffer with 150 mM of NaCl at pH 7.2. This buffer is filtered through a pore size of 0.1 μm and degassed for 15 minutes before use. The chromatography system is equilibrated at a flow rate of 0.5 ml/min until the signal in Dawn 8 stabilized at around 0.3 detector voltage units. The UV-visible detector is set at 220 nm and the column is oven equilibrated to 25ºC. The elution mode is isocratic and the run time is 40 minutes. The volume of sample injected ranged from 50 to 100 μl. The % area of the exosome peak *vs.* all other peaks is integrated from the UV-visible detector. The hydrodynamic radius, R_h is computed by the QELS and Dawn 8 detectors. The highest count rate (Hz) at the peak apex is taken as the R_h.

Peaks of the separated components visualized at 220 nm are collected as fractions for further characterization studies.

Example 34. Materials and Methods: Sucrose Gradient Density Equilibrium Centrifugation

For sucrose gradient density equilibrium centrifugation, 14 sucrose solutions with concentrations from 22.8-60% (w/v) are prepared. The most concentrated solution is

layered at the bottom of SW60Ti ultracentrifuge tube (Beckman Coulter Inc., Fullerton CA, USA), followed by the next highest sucrose concentration. CM is carefully loaded on top before ultracentrifuged for 16.5 hours at 200,000X g, 4°C in a SW60Ti rotor (Beckman Coulter Inc.). 16 fractions are collected from top to the bottom of the sucrose gradient. The densities of all the sucrose fractions are calculated using a microbalance, and consolidated intoto 13 fractions. For some CM, the CM is pretreated with a cell lysis buffer (Cell Extraction Buffer, Biovision, www.BioVision.com) before being loaded on sucrose gradient density equilibrium centrifugation. The lysis buffer is added to CM in a 1:1 volume ratio with cocktail of protease inhibitors (Halt Protease Inhibitor Cocktail, EDTA-Free, Thermo Scientific , www.thermofisher.com). The mixture is incubated for 30 min at room temperature with gentle shaking.

Example 35. Materials and Methods: Protein Quantitation

Protein concentration of CM is quantified using NanoOrange Protein Quantification kit (Invitrogen) according to the manufacturer's instructions.

Example 36. Materials and Methods: SDS-PAGE and Western Blot Analysis

Total proteins of CM are separated on polyacrylamide gels, before transfer to a nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden). The membrane is blocked, incubated with mouse antibodies against human CD9, CD81, SOD-1, pyruvate kinase, Alix, Tsp-1 followed by horseradish peroxidase-coupled secondary antibodies against the mouse primary antibody. The blot is then incubated with a chemiluminescent HRP substrate to detect bound primary antibody, and therefore the presence of the antigen.

Example 37. Materials and Methods: Sphingomyelin, Phosphatidylcholine and Cholesterol Assay

Cholesterol, sphingomyelin and phosphatidylcholine concentrations in two independent preparations of CM and pellet from the ultracentrifugation of CM at 100,000 xg for 2 hours at 4 °C are measured using commercially available assay kits. Cholesterol is measured using Amplex® Red Cholesterol Assay kit (Molecular Probes, USA), sphingomyelin by the Sphingomyelin Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) and phosphatidylcholine is measured using the Phosphatidylcholine Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA).

Example 38. Materials and Methods: Limited Trypsinization of Conditioned Medium

CM is treated with/without triton x or lysis buffer for 30 min at 4 °C with gentle shaking. Proteolytic digestion is allowed to carry out by adding trypsin to the treated CM for 3 seconds to 20 minutes at room temperature with gentle shaking. The digestion is stopped using a trypsin inhibitor, PMSF.

Example 39. Materials and Methods: miRNA Microarray Analysis

Two biological replicates of total cellular RNA from MSCs and two biological replicates of secreted RNA from CM are analysed by miRNA microarray. The hybridization and data analysis is outsourced to LC Sciences, LLC (www.LCsciences.com). The chip contained probes for miRNA transcripts listed in Sanger miRBase Release 10.1 (http://www.sanger.ac.uk/Software/Rfam/mirna/).

Example 40. Results: Cardoprotective Secretion Contains Exosome-associated Proteins that Form Multiprotein Complexes

To identify the active component, we have previously fractionated the CM by ultrafiltration through membranes with different MWCO. It is shown that when CM is filtered through a membrane with MWCO of 1000 kDa, the filtrate is not protective. However, CM that is concentrated by ~125 times against a similar membrane is cardioprotective in a mouse model of ischemia/reperfusion injury. In summary, filteration through filters with a MWCO smaller than 0.2μm such as100kDa, 300kDa, 500kDa or 1000kDa are not cardioprotective (Figure 14) but CM that is concentrated against a 1000 kDa membrane (Timmers et al., 2008) or a 100 kDa membrane is cardioprotective (Figure 14). These observations suggested then that the active fraction consisted of large complexes of >1000 kDa or having a diameter of 50-100 nm. Based on the size range of the particles, we postulated that the particles in the CM are exosomes. Exosomes are formed from multivesicular bodies (Fevrier and Raposo, 2004; Keller et al., 2006) with a bilipid membrane that has the same orientation as plasma membrane. They are known to be produced by many cell types and are thought to be important in intercellular communications. Exosomes have diameters of 40-100 nm. Exosomes have been shown to be secreted by many cell types and the protein composition of these exosomes appeared to be cell specific. However, some proteins such as CD9, pyruvate kinase and alix appear to

be commonly expressed in the exosomes (Sze et al., 2007). We have previously identified about 201 proteins in the secretion (Sze et al., 2007).

Here we extended the list to 793 proteins (Table E2) by making modifications to our previously described methodology in our proteomic analysis as detailed in the Materials and Methods. The 793 contained many of the exosome-associated proteins such as CD9, CD81, Alix, TSP-1, SOD-1 and pyruvate kinase (Olver and Vidal, 2007). We confirmed the presence of these proteins in the secretion by western blot analysis (Lane1, Figure 15). Co-immunoprecipitation of CD81, CD9 and Alix supported their association with an exosome and the presence of exosome in the secretion. TSP-1, SOD-1 and pyruvate kinase did not co-immunoprecipitated with CD81, suggesting that these proteins are not present in CD81+ exosomes or not present in exosomes at all. (Figure 15).

Table E2 (below). Alphabetical list of 739 unique gene products identified by LC-MS/MS and antibody array

02-Sep	BPNT1	COL5A3	FAM3C	HINT1	ITGB4BP	MYH9	PPIA	RPLP2	THBS2
07-Sep	BTD	COL6A1	FAM49B	HIST1H4	K-ALPHA-1	MYL6	PPIB	RPS10	THOP1
AARS	C14orf141	COL6A2	FAM62A	HIST1H4A	KPNB1	NAGK	PPP2R1A	RPS15A	THY1
ACAA2	C19orf10	COL6A3	FBLN1	HIST1H4B	KRT1	NANS	PPP2R4	RPS16	TIMP1
ACAT2	C1orf58	COL7A1	FBLN5	HIST1H4C	KRT14	NARS	PPP5C	RPS19	TIMP2
ACO1	C1orf78	COPA	FBN1	HIST1H4D	KRT2	NEDD8	PPP6C	RPS2	TIMP3
ACTB	C1QBP	COPG	FBN2	HIST1H4E	KRT27	NEFM	PRDX1	RPS20	TKT
ACTC1	C1R	COPS3	FDPS	HIST1H4F	KRT4	NIT2	PRDX2	RPS23	TLN1
ACTN1	C1S	COPS4	FGF16	HIST1H4H	KRT5	NME1	PRDX3	RPS3	TMOD2
ACTN2	C21orf33	COPS8	FGFRL1	HIST1H4I	KRT6L	NPC2	PRDX4	RPS4X	TMOD3
ACTN3	CALR	CORO1B	FH	HIST1H4J	KRT7	NPEPPS	PRDX5	RPS5	TNC
ACTN4	CAND1	CORO1C	FKBP10	HIST1H4K	KRT75	NPM1	PRDX6	RPS7	TNFRSF11B
ACTR1A	CAP1	COTL1	FKBP1A	HIST1H4L	KRT77	NQO1	PRG1	RPS8	TNFRSF12A
ACTR1B	CAP2	CRIP2	FKBP3	HIST2H2AA3	KRT9	NRP1	PRKACA	RPS9	TNFSF12
ACTR2	CAPG	CS	FLNA	HIST2H2AA4	KRTHB4	NRP2	PRKCSH	RPSA	TNPO1
ACTR3	CAPN1	CSE1L	FLNB	HIST2H4A	LAMA4	NT5E	PRNP	RSU1	TP53I3
ACTR3B	CAPN2	CSRP1	FLNC	HIST2H4B	LAMB1	NUCB1	PROCR	RTN4	TPI1
ADAM9	CAPZA1	CSRP2	FLRT2	HIST4H4	LAMC1	OLFML3	PROSC	S100A11	TPM1
ADSL	CAPZA2	CST3	FLT1	HLA-A	LANCL1	P4HA1	PRSS23	S100A16	TPM2
ADSS	CAPZB	CTGF	FN1	HLA-B	LAP3	P4HB	PRSS3	SARS	TPM3
AEBP1	CARS	CTHRC1	FSCN1	HMX1	LASP1	PABPC1	PSAP	SDC4	TPM4
AGA	CBR1	CTSB	FSTL1	HNRPA1	LDHA	PABPC4	PSAT1	SDCBP	TRAP1
AGRN	CBR3	CTSD	FSTL5	HNRPA1L-2	LDHAL6B	PAFAH1B1	PSMA1	SEC22B	TRHDE
AHCY	CCBL2	CTSZ	FTL	HNRPA2B1	LDHB	PAFAH1B2	PSMA2	SEC23A	TROVE2
AK1	CCDC19	CXCL1	G6PD	HNRPC	LEPRE1	PAFAH1B3	PSMA3	SEC31A	TSKU
AK2	CCL18	CXCL12	GALNT2	HNRPCL1	LGALS1	PAICS	PSMA6	SEMA3C	TUBA1A
AKR1A1	CCL2	CXCL16	GALNT5	HNRPD	LGALS3	PAM	PSMA7	SEMA7A	TUBA6
AKR1B1	CCL7	CXCL2	GANAB	HNRPDL	LGALS3BP	PAPPA	PSMB1	SERPINB	TUBA8

								1	
ALCAM	CCN4	CXCL9	GAPDH	HNRPH2	LMNA	PARK7	PSMB2	SERPINB6	TUBB
ALDH2	CCR4	CYCS	GARS	HNRPK	LOC196463	PARP1	PSMB3	SERPINE1	TUBB2C
ALDH7A1	CCR5	D4ST1	GAS6	HNRPL	LOC283523	PARVA	PSMB4	SERPINE2	TUBB3
ALDOA	CCT2	DAG1	GBA	HNRPR	LOC347701	PCBP1	PSMB5	SERPINF1	TUBB4
ALDOC	CCT3	DCI	GBE1	HNRPU	LOC646821	PCBP2	PSMD11	SERPINH1	TUBB6
ANGPT4	CCT4	DCN	GDF1	HNT	LOC649125	PCDH18	PSMD13	SERPINI2	TUBB8
ANP32B	CCT5	DDAH2	GDF11	HSP90AB1	LOC653214	PCDHGB6	PSMD5	SFRP1	TWF1
ANXA1	CCT6A	DDB1	GDF15	HSP90B1	LOC654188	PCK2	PSMD6	SFRP4	TXN
ANXA2	CCT7	DDT	GDF3	HSPA1A	LOC728378	PCMT1	PSMD7	SH3BGRL3	TXNL5
ANXA5	CCT8	DDX17	GDF5	HSPA1B	LOXL2	PCNA	PSME1	SIL1	TXNRD1
ANXA6	CD109	DES	GDF8	HSPA1L	LRP1	PCOLCE	PSME2	SLC1A5	UBE1
AP1B1	CD248	DKK1	GDI1	HSPA4	LTA	PDCD6IP	PTBP1	SLC3A2	UBE2L3
AP1S1	CD44	DLD	GDI2	HSPA5	LTA4H	PDGFA	PTK7	SND1	UBE2N
AP2A1	CD59	DNAJC3	GLO1	HSPA6	LTB	PDGFC	PTPRCAP	SNRPD1	UBE2V1
AP2A2	CD81	DPP3	GLRX	HSPA8	LTB4DH	PDGFRB	PTX3	SNRPE	UBE3B
AP2B1	CD9	DPYSL2	GLT8D3	HSPB1	LTBP1	PDIA3	PURA	SOD1	UCHL1
AP3B1	CDC37	DPYSL3	GLUD1	HSPD1	LTBP2	PDIA4	PXDN	SPARC	UCHL3
APEX1	CDC42	DSTN	GM2A	HSPE1	LUM	PDIA6	PYCR1	SPOCK	UGDH
API5	CDH11	DYNLL1	GNPDA1	HSPG2	M6PRBP1	PDLIM1	PYGB	SPTAN1	UGP2
APOA1BP	CDH13	ECHS1	GNPNAT1	HSPH1	MACF1	PDLIM5	QARS	SPTBN1	UROD
APOE	CDH2	ECM1	GOT1	HTRA1	MADH4	PDLIM7	QPCT	SPTBN4	USP14
APP	CFL1	EEF1A1	GOT2	IDH1	MAP1B	PEPD	QSCN6	SRP9	USP5
APRT	CFL2	EEF1A2	GPC1	IFNG…	MAPK1	PFN1	RAB11B	SRPX	VARS
ARCN1	CHID1	EEF1B2	GPC5	IGF2R	MAPRE1	PFN2	RAB1A	SRPX2	VASN
ARHGAP1	CHRDL1	EEF1G	GPI	IGFBP2	MAT2A	PGCP	RAB6A	SSB	VAT1
ARHGDIA	CLEC11A	EEF2	GREM1	IGFBP3	MAT2B	PGD	RAC1	ST13	VCL
ARPC1A	CLIC1	EFEMP2	GRHPR	IGFBP4	MCTS1	PGK1	RAN	ST6GAL2	VCP
ARPC1B	CLIC4	EIF2S3	GRN	IGFBP5	MDH1	PGK2	RANBP5	STAT1	VEGFC
ARPC2	CLSTN1	EIF3S9	GSN	IGFBP7	MDH2	PGLS	RARRES2	STC1	VIL2
ARPC3	CLTC	EIF4A1	GSR	IGKC	MFAP4	PGM1	RARS	STC2	VIM
ARPC4	CLTCL1	EIF4A2	GSS	IL13	MGAT5	PGRMC2	RBMX	STIP1	VPS26A
ARTS-1	CLU	EMILIN1	GSTK1	IL15	MIF	PHGDH	RHOA	SULF1	VPS35
ATIC	CMPK	EML2	GSTO1	IL15RA	MMP1	PHPT1	RNASE4	SVEP1	VTN
ATP5B	CNDP2	ENO1	GSTP1	IL1RAP	MMP10	PICALM	RNH1	SYNCRIP	WARS
ATP6AP1	CNN2	ENO2	GTPBP9	IL2	MMP14	PKM2	RNPEP	TAGLN	WDR1
ATP6AP2	CNN3	ENO3	GZMA	IL21R	MMP2	PLAU	RPL10A	TAGLN2	WNT5A
ATP6V1B2	COL12A1	EPPK1	H2AFY	IL3	MRC2	PLEC1	RPL11	TALDO1	WNT5B
ATP6V1G2	COL18A1	EPRS	HADH	IL6	MRLC2	PLEKHC1	RPL12	TARS	XPO1
B2M	COL1A1	ESD	HARS	IL6ST	MSN	PLOD1	RPL14	TCN2	YKT6
B4GALT1	COL1A2	ETF1	HARS2	IL8	MTAP	PLOD2	RPL18	TCP1	YWHAB
BASP1	COL2A1	ETFB	hCG_1641617	ILF2	MTPN	PLOD3	RPL22	TFPI	YWHAE

BAT1	COL3A1	ETHE1	hCG_20 23776	ILF3	MVP	PLS1	RPL30	TGFB1	YWHAG
BBS1	COL4A1	EXT1	HEXA	INHBA	MXRA5	PLS3	RPL5	TGFB2	YWHAH
BCAT1	COL4A2	FAH	HEXB	IQGAP1	MXRA8	PLSCR3	RPL7	TGFBI	YWHAQ
BGN	COL5A1	FAHD1	HGF	ISOC1	MYH11	POSTN	RPLP0	THBS	YWHAZ
BLVRA	COL5A2	FAM129 B	HIBCH	ITGA2	MYH14	PPCS	RPLP1	THBS1	

Table E2. Proteomic profile of CM as determined by LC MS/MS and antibody arrays. Four independent samples were analyzed. Each protein in the table was detected in at least 3 of 4 samples.

Of the above proteins in Table E2, TIMP1, TIMP2, TNFRSF11B, LGALS3, ALCAM, DCN, SFRP1, GDF15, PDGFC, PTX3, LTBP1, IGFBP2, GREM1, IGFBP7, MIF, MMP1, PLAU, INHBA and THBS1 were identified by LC MS/MS and antibody array. , PPIA, HIST1H4, PPIB, HIST1H4A, HIST1H4B, HIST1H4C, HIST1H4D, HIST1H4E, HIST1H4F, HIST1H4H, HIST1H4I, HIST1H4J, HIST1H4K, HIST1H4L, HIST2H2AA3, HIST2H2AA4, HIST2H4A, HIST2H4B, HIST4H4, HLA-A, HLA-B, SDCBP, TUBA1A, TUBA6, TUBA8, GAPDH, TUBB, TUBB2C, TUBB3, TUBB4, TUBB6, TUBB8, HSP90AB1, ANXA1, HSP90B1, ANXA2, ANXA5, ANXA6, PDCD6IP, CD9, CFL1, CLTC, ENO1, PKM2, MSN, and YWHAG are identified by LC MS/MS and at least 4 studies on exosomes secreted by cultured cells. FGF16, FGFRL1, TNFRSF12A, TNFSF12, CXCL1, CCL18, CXCL12, CCL2, CXCL16, CCL7, CXCL2, CCN4, CXCL9, CCR4, CCR5, ANGPT4, GDF1, GDF11, SFRP4, GDF3, GDF5, GDF8, DKK1, LTA, PDGFA, LTB, MADH4, IFNG…, GPC5, IGF2R, CHRDL1, GRN, VEGFC, IL13, IL15, EML2, IL15RA, IL1RAP, MMP10, IL2, GZMA, IL21R, IL3, IL6, IL6ST, IL8, HGF and THBS are identified by antibody array. The remaining proteins are identified by LC MS/MS.

Example 41. Results: Exosome-associated Proteins are Localized in Phospholipid Vesicles

To verify the presence of exosome in the CM, the CM is ultracentrifuged at 200,000g for two hours. There is a >200 fold enrichment of CD9 an exosome-associated protein in the pellet with no detectable level of CD9 in the supernatant (Figure 16). Ultracentrifugation at 100,000g for one hour is not sufficient to sediment all the CD9 (Figure 16). Filtration of the CM through a filter with a MWCO of 500 kDa followed by centrifugation of either the filtrate or retentate at 200,000g for 2hours generated a pellet in the retentate fraction (Figure 16). CD9 which had MW of 19 kDa respectively are highly

enriched in this pellet. However, this pellet did not confer any cardioprotection in a mouse model of ischemia/reperfusion injury and we postulated that this is due to the need for vigorous vortexing and pipeting to resuspend the pellet. We observed that only a fraction of CD9 sedimented at 100,000 g and 200, 000g for one hour and most sedimented at 200,000 g for two hours. A small fraction sedimented at 200,000 g for four hours. Together , these observations support our hypothesis that the active component is relatively large complex that can be sedimented by ultracentrifugation.

To confirm that the exosome-associated proteins are indeed in exosomes i.e. phospholipid vesicles, the CM is fractionated on a sucrose density gradient by equilibrium ultracentrifugation. Like lipid vesicles, the density of exosomes ranges from 1.13 g ml^{-1} to 1.19 g ml−1 and float on sucrose gradients. Flotation on sucrose gradients readily separate exosomes from contaminating material such as protein aggregates or nucleosomal fragmentst (Thery et al., 2002). Fractions from the sucrose gradient are then analysed for the presence CD9, CD81, Tsp1, SOD-1 and pyruvate kinase along the gradient (Figure 17A). A notable feature is that the proteins did not sediment to the expected density of proteins that is correlated to their molecular weight. To determine if these apparent densities are due to the proteins being contained in lipid vesicles, CM is treated with a cell lysis buffer (Figure 17B) before being fractionated on a sucrose density gradient. This pre-treatment with a plasma membrane solubilization reagent restored each of the apparent densities to the expected density of proteins that correlated to their molecular weight. Therefore, the exosome-associated proteins are localized in lipid vesicles, consistent with our exosome hypothesis.

To confirm that there are lipid vesicles in the CM, the concentration of sphingomyelin and phosphatidylcholine, the major phospholipids of the plasma membrane, and cholesterol is determined (Figure 17C). As expected, the relative concentration of these lipids per μg protein is higher in the CM relative to the non-conditioned medium. Furthermore, ultracentrifugation of the CM at 200,000 g for 2 hours significantly increased the concentration of the lipids (Figure 17C).

Example 42. Results: Exosomal Proteins are Either Membrane Bound or Encapsulated

As the exosome-associated proteins include many known membrane proteins such as CD9 and cytosolic proteins such as SOD1, we therefore determined if these proteins are

similarly localized on the lipid membrane and lumen of the vesicles. CM is subjected to limited trypsinization over time (Figure 18A). CD9 which has a similar MW as SOD1 is relatively more susceptible to trypsin digest than SOD1. Digestion of SOD1 is observed only after more than 50% of CD9 had been digested (Figure 18A). Unlike tryptic digestion of SOD1 which generated no detectable intermediates, tryptic digestion of CD9 generated three tryptic peptide intermediates, suggesting that CD9 has domains with different trypsin-sensitivity. Based on the length of peptide intermediates and known tryptic sites of CD9, the three susceptible tryptic peptide intermediates are mapped to the transmembrane or cytoplasmic domains. This suggested that the known extra-cytoplasmic domain of cellular CD9 is similarly exposed on the secreted CD9 and therefore trypsin-sensitive while the trans-membrane and cytoplasmic domain is not exposed and is therefore relatively resistant to tryptic digest. Together, these observations suggested that CD9, a known membrane protein is also membrane-bound in the exosome and is oriented in the same direction as CD9 in the plasma membrane while cytosolic SOD-1 is localized in the lumen and could be digested only when the integrity of the membrane is compromised by the digestion of membrane proteins.

Example 43. Results: Presence of Lipid Vesicle Encapsulated RNA in the Secretion of MSCs

It is previously reported that RNAs are secreted by cells in exosomes (Smalheiser, 2007; Taylor and Gercel-Taylor, 2008; Valadi et al., 2007). To determine if RNA is present in the cardioprotective secretion, CM is extracted for RNA by Trizol to yield 5- 6 ug RNA per mg protein. When separated on a glyoxal-agarose gel (Figure 19A) or an urea-PAGE (Figure 19B), the RNA contained undetectable level of 18S and 28S ribosomal RNA with most RNAs being <300 nt. To determine if the stability of the RNA in the secretion is due to its encapsulation within as a phospholipid vesicle as observed for the proteins, the CM is treated with RNase before being extracted for RNA. The RNA yield and the size distribution are similar to that of untreated CM (Figure 19C), suggesting that the secreted RNAs are protected from RNAse degradation. We next tested the possibility that the RNAs are protected by a lipid membrane, analogous to a cell membrane by treating the CM with a SDS-based cell lysis buffer, cylcodextrin or phospholipase A2. After treatment with one of the four reagents, the CM is exposed to RNAse and then extracted for RNA. Pretreatment with a SDS-based cell lysis buffer

resulted in a complete loss of RNAs while treatment with cylcodextrin or phospholipase A2 led to partial degradation and loss of RNAs. These observations suggested that the RNA is protected from RNAse activity by a cholesterol-rich phospholipid membrane such that the membrane is readily dissolved or compromised by a SDS-based cell lysis buffer, a detergent such as TritonX-100 that dissolves lipids, cylcodextrin that chelates and extracts cholesterol or degradation by phospholipase D. We also observed that RNAs of ~70-100nt are more sensitive to RNAse III activity than those of smaller MW, suggesting that the larger RNAs are double stranded (Figure 19D).

Example 44. Results: Secreted RNAs are Sequestered in Vesicles

Since the RNAs are shown to be in lipid vesicles, we next determined the buoyant density of these vesicles using sucrose gradient equilibrium ultracentrifugation. CM, CM pretreated with lysis buffer or a set of RNA MW markers is loaded onto a sucrose density gradient and ultracentrifuged as described in figure 4a, b. The gradients are then removed in thirteen fractions and each fraction is then extracted for RNA. The secreted RNAs equilibrated at a density of 1.074-1.1170 g/ml (Figure 20). In contrast, RNA MW markers exhibited a buoyant density of 1.115-1.1170g/ml and pretreatment of the CM with lysis buffer before centrifugation caused an increase in density of the secreted RNA to that of RNA MW markers i.e. 1.115-1.145 g/ml (Figure 20C). These observations are therefore consistent with the RNAs being encapsulated in a lipid vesicle and thus had an apparent density that is much lower than soluble RNA. Pretreatment of the CM with lysis buffer released the RNA and resulted in the RNA sedimenting at the density of RNA markers.

Example 45. Results: RNA-containing Vesicles are not in CD81 Containing Exosomes

As shown above, CD9, CD81 and Alix are co-immunoprecipitated by anti CD81 antibodies. Here we tested if the RNA also immunoprecipitate with CD81. After immunoprecipitation, the RNA is not present in the precipitate but remained in the supernatant (Figure 21). Therefore, the secreted RNAs are not sequestered in CD81+, CD81+ CD9+, or CD81+ CD9+ Alix+ vesicles.

Example 46. Results: Secreted RNA Contain microRNAs that Include pre-miRNAs

It has been reported that exosomes contain microRNAs (Smalheiser, 2007; Taylor and Gercel-Taylor, 2008; Valadi et al., 2007) and as most of the RNAs in the CM are less than 300 nt, we tested the RNA from the MSC and their CM for the presence of

microRNAs (miRNAs) by performing a microarray hybridization. 149 miRNA are detected in MSC and 63 are detected in the CM (Figure 22A, Table E3 below).

hsa-let-7a	hsa-miR-24-2*	hsa-miR-98	hsa-miR-149*	hsa-miR-214	hsa-miR-484
hsa-let-7b	hsa-miR-25	hsa-miR-99a	hsa-miR-151-3p	hsa-miR-221	hsa-miR-491-5p
hsa-let-7c	hsa-miR-26a	hsa-miR-99b	hsa-miR-151-5p	hsa-miR-222	hsa-miR-503
hsa-let-7d	hsa-miR-26b	hsa-miR-100	hsa-miR-152	hsa-miR-320	hsa-miR-505*
hsa-let-7e	hsa-miR-27a	hsa-miR-103	hsa-miR-155	hsa-miR-324-5p	hsa-miR-532-5p
hsa-let-7f	hsa-miR-27b	hsa-miR-106a	hsa-miR-181a	hsa-miR-328	hsa-miR-574-3p
hsa-let-7g	hsa-miR-27b*	hsa-miR-106b	hsa-miR-181a*	hsa-miR-330-3p	hsa-miR-574-5p
hsa-let-7i	hsa-miR-28-3p	hsa-miR-107	hsa-miR-181a-2*	hsa-miR-331-3p	hsa-miR-575
hsa-miR-10a	hsa-miR-28-5p	hsa-miR-125a-3p	hsa-miR-181b	hsa-miR-335	hsa-miR-584
hsa-miR-15a	hsa-miR-29a	hsa-miR-125a-5p	hsa-miR-181c	hsa-miR-342-3p	hsa-miR-612
hsa-miR-15b	hsa-miR-29c	hsa-miR-125b	hsa-miR-181d	hsa-miR-345	hsa-miR-625
hsa-miR-16	hsa-miR-30a	hsa-miR-126	hsa-miR-185	hsa-miR-361-5p	hsa-miR-629
hsa-miR-17	hsa-miR-30a*	hsa-miR-128	hsa-miR-186	hsa-miR-362-3p	hsa-miR-638
hsa-miR-18a	hsa-miR-30b	hsa-miR-130a	hsa-miR-187*	hsa-miR-362-5p	hsa-miR-663
hsa-miR-18b	hsa-miR-30c	hsa-miR-130b	hsa-miR-191	hsa-miR-365	hsa-miR-671-5p
hsa-miR-19b	hsa-miR-30d	hsa-miR-132	hsa-miR-191*	hsa-miR-374b	hsa-miR-708
hsa-miR-20a	hsa-miR-30e	hsa-miR-137	hsa-miR-192	hsa-miR-421	hsa-miR-744
hsa-miR-20b	hsa-miR-30e*	hsa-miR-140-3p	hsa-miR-193a-5p	hsa-miR-423-5p	hsa-miR-766
hsa-miR-21	hsa-miR-31	hsa-miR-143	hsa-miR-195	hsa-miR-424	hsa-miR-768-3p
hsa-miR-22	hsa-miR-31*	hsa-miR-145	hsa-miR-197	hsa-miR-424*	hsa-miR-768-5p
hsa-miR-22*	hsa-miR-34a	hsa-miR-145*	hsa-miR-199a-3p	hsa-miR-425	hsa-miR-769-5p
hsa-miR-23a	hsa-miR-34a*	hsa-miR-146a	hsa-miR-199a-5p	hsa-miR-425*	hsa-miR-877
hsa-miR-23a*	hsa-miR-92a	hsa-miR-146b-5p	hsa-miR-199b-5p	hsa-miR-454	hsa-miR-923

hsa-miR-23b	hsa-miR-92b	hsa-miR-148b	hsa-miR-210	hsa-miR-455-3p	hsa-miR-940
hsa-miR-24	hsa-miR-93	hsa-miR-149	hsa-miR-212	hsa-miR-483-5p	
hsa-let-7b*	hsa-miR-124	hsa-miR-296-5p	hsa-miR-765	hsa-miR-1228	hsa-miR-1238
hsa-let-7d*	hsa-miR-150*	hsa-miR-493*	hsa-miR-933	hsa-miR-1234	
hsa-miR-122	hsa-miR-198	hsa-miR-572	hsa-miR-1224-5p	hsa-miR-1237	

Table E3. List of miRNAs in MSC and CM as determined by microarray hybridization.

47 miRNAs are present in both MSC and CM. These are: hsa-let-7a, hsa-miR-149*, hsa-miR-214, hsa-let-7b, hsa-miR-221, hsa-let-7c, hsa-miR-26a, hsa-miR-151-5p, hsa-miR-222, hsa-let-7d, hsa-miR-100, hsa-miR-320, hsa-let-7e, hsa-miR-103, hsa-let-7f, hsa-miR-181a, hsa-miR-574-3p, hsa-miR-574-5p, hsa-let-7i, hsa-miR-107, hsa-miR-575, hsa-miR-125a-5p, hsa-miR-125b, hsa-miR-361-5p, hsa-miR-638, hsa-miR-663, hsa-miR-191, hsa-miR-671-5p, hsa-miR-132, hsa-miR-191*, hsa-miR-193a-5p, hsa-miR-423-5p, hsa-miR-21, hsa-miR-31, hsa-miR-143, hsa-miR-22, hsa-miR-145, hsa-miR-23a, hsa-miR-146a, hsa-miR-425*, hsa-miR-92a, hsa-miR-923, hsa-miR-23b, hsa-miR-940, hsa-miR-24, hsa-miR-149 and hsa-miR-483-5p.

16 miRNAs are detectable in the CM but are present at below detection level in MSCs. These are: hsa-let-7b*, hsa-miR-124, hsa-miR-296-5p, hsa-miR-765, hsa-miR-1228, hsa-miR-1238, hsa-let-7d*, hsa-miR-150*, hsa-miR-493*, hsa-miR-933, hsa-miR-1234, , hsa-miR-122, hsa-miR-198, hsa-miR-572, hsa-miR-1224-5p an dhsa-miR-1237.

The following miRNAs are present in MSC only: hsa-miR-24-2*, hsa-miR-98, hsa-miR-484, hsa-miR-25, hsa-miR-99a, hsa-miR-151-3p, hsa-miR-491-5p, hsa-miR-99b, hsa-miR-503, hsa-miR-26b, hsa-miR-152, hsa-miR-505*, hsa-miR-27a, hsa-miR-155, hsa-miR-324-5p, hsa-miR-532-5p, hsa-miR-27b, hsa-miR-106a, hsa-miR-328, hsa-let-7g, hsa-miR-27b*, hsa-miR-106b, hsa-miR-181a*, hsa-miR-330-3p, hsa-miR-28-3p, hsa-miR-181a-2*, hsa-miR-331-3p, hsa-miR-10a, hsa-miR-28-5p, hsa-miR-125a-3p, hsa-miR-181b, hsa-miR-335, hsa-miR-584, hsa-miR-15a, hsa-miR-29a, hsa-miR-181c, hsa-miR-342-3p, hsa-miR-612, hsa-miR-15b, hsa-miR-29c, hsa-miR-181d, hsa-miR-345, hsa-miR-625, hsa-miR-16, hsa-miR-30a, hsa-miR-126, hsa-miR-185, hsa-miR-629, hsa-miR-17,

hsa-miR-30a*, hsa-miR-128, hsa-miR-186, hsa-miR-362-3p, hsa-miR-18a, hsa-miR-30b, hsa-miR-130a, hsa-miR-187*, hsa-miR-362-5p, hsa-miR-18b, hsa-miR-30c, hsa-miR-130b, hsa-miR-365, hsa-miR-19b, hsa-miR-30d, hsa-miR-374b, hsa-miR-708, hsa-miR-20a, hsa-miR-30e, hsa-miR-137, hsa-miR-192, hsa-miR-421, hsa-miR-744, hsa-miR-20b, hsa-miR-30e*, hsa-miR-140-3p, hsa-miR-766, hsa-miR-195, hsa-miR-424, hsa-miR-768-3p, hsa-miR-31*, hsa-miR-197, hsa-miR-424*, hsa-miR-768-5p, hsa-miR-22*, hsa-miR-34a, hsa-miR-145*, hsa-miR-199a-3p, hsa-miR-425, hsa-miR-769-5p, hsa-miR-34a*, hsa-miR-199a-5p, hsa-miR-877, hsa-miR-23a*, hsa-miR-146b-5p, hsa-miR-199b-5p, hsa-miR-454, hsa-miR-92b, hsa-miR-148b, hsa-miR-210, hsa-miR-455-3p, hsa-miR-93, hsa-miR-212.

The microarray analysis also indicated that the CM contained significant levels of anti-guide miRNA (denoted with an asterisk). For example, the relative ratios of let7b to let7b*, let7d to let7d* and miR-191 to miR-191* in CM are much reduced compared to that in the MSCs (Figure 22B). In cells, cleavage of the stem-loop pre-miRNA generates the mature guide miRNA and the anti-guide miRNA*. The latter is usually degraded in the cell. One possible explanation for the low ratio of guide miRNA to anti-guide miRNA is that microRNAs in the secretion are pre-miRNAs, and not mature RNAs. Consistent with the possibility is our observations that the 70-100nt RNAs are double-stranded (Figure 19D). To confirm this finding, real time PCR analysis of reverse transcribed RNAs are performed using primers specific for guide mRNA and pre-miRNA with or without prior treatment with RNAse III. RNAse III treatment will confirm the presence of pre-miRNA by degrading the pre-miRNA and render it undetectable by RT-PCR.

Example 47. Results: Cardioprotective Secretion Contains only Particles with Hydrodynamic Radius of 45-55 nm in the Detectable Range of 1-1000 nm

To confirm that the secretion contained large complexes, CM and NCM is first separated by size exclusion on a HPLC (Figure 13) and each eluting peak as measured by absorbance at 220 nm is then examined for dynamic light scattering (DLS) using a quasi-elastic light scattering (QELS) detector.

Only one eluting peak with a retention time of 11-13 minutes exhibited dynamic light scattering and the r_h of particles in this peak is about 45-55 nm. The other eluting peaks with a retention time of 13-16 and 17-19 minutes did not exhibit dynamic light scattering. Since the r_h detection range of DLS is from 1 to 1000 nm in and the diameter of

a DNA alpha helix is 2 nm, globular proteins is 1-10 nm, nuclear pore (50 nm), large virus (100 nm) and mitochondrion is 3 μM, DLS is therefore capable of detecting most biological particles.

Based on our observation that that cardioprotection is associated with a fraction that has MW of >1000 kDa, we hypothesize that the eluting peak at a retention time of 12 minutes is the active component.

To confirm this, this eluting peak are harvested and tested in a mouse model of myocardial ischemia/reperfusion injury as previously described above and in (Timmers et al., 2008).

REFERENCES (INCLUDING FOR EXAMPLES 1 TO 18)

1. Lewis EF, Moye LA, Rouleau JL, et al. Predictors of late development of heart failure in stable survivors of myocardial infarction: the CARE study. *J Am Coll Cardiol.* Oct 15 2003;42(8):1446-1453.

2. Kleiman NS, White HD, Ohman EM, et al. Mortality within 24 hours of thrombolysis for myocardial infarction. The importance of early reperfusion. The GUSTO Investigators, Global Utilization of Streptokinase and Tissue Plasminogen Activator for Occluded Coronary Arteries. *Circulation.* Dec 1994;90(6):2658-2665.

3. Saraste A, Pulkki K, Kallajoki M, et al. Apoptosis in human acute myocardial infarction. *Circulation.* Jan 21 1997;95(2):320-323.

4. Smits AM, van Vliet P, Hassink RJ, et al. The role of stem cells in cardiac regeneration. *J Cell Mol Med.* Jan-Mar 2005;9(1):25-36.

5. Menasche P, Hagege AA, Vilquin JT, et al. Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. *J Am Coll Cardiol.* Apr 2 2003;41(7):1078-1083.

6. Perin EC, Dohmann HF, Borojevic R, et al. Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. *Circulation.* May 13 2003;107(18):2294-2302.

7. Shake JG, Gruber PJ, Baumgartner WA, et al. Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. *Ann Thorac Surg.* Jun 2002;73(6):1919-1925; discussion 1926.

8. Strauer BE, Brehm M, Zeus T, et al. Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation.* Oct 8 2002;106(15):1913-1918.

9. Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res.* Jul 9 2004;95(1):9-20.

10. Minguell JJ, Erices A. Mesenchymal stem cells and the treatment of cardiac disease. *Exp Biol Med (Maywood).* Jan 2006;231(1):39-49.

11. Zimmet JM, Hare JM. Emerging role for bone marrow derived mesenchymal stem cells in myocardial regenerative therapy. *Basic Res Cardiol.* Nov 2005;100(6):471-481.

12. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. *J Cell Biochem.* Aug 1 2006;98(5):1076-1084.

13. Gnecchi M, He H, Liang OD, et al. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nat Med.* Apr 2005;11(4):367-368.

14. Gnecchi M, He H, Noiseux N, et al. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *Faseb J.* Apr 2006;20(6):661-669.

15. Lian Q, Lye E, Suan Yeo K, et al. Derivation of clinically compliant MSCs from CD105+, CD24- differentiated human ESCs. *Stem Cells.* Feb 2007;25(2):425-436.

16. Sze SK, de Kleijn DP, Lai RC, et al. Elucidating the secretion proteome of human ESC-derived mesenchymal stem cells. *Mol Cell Proteomics.* Jun 11 2007.

17. Birnbaum Y, Hale SL, Kloner RA. Differences in reperfusion length following 30 minutes of ischemia in the rabbit influence infarct size, as measured by triphenyltetrazolium chloride staining. *J Mol Cell Cardiol.* Feb 1997;29(2):657-666.

18. Timmers L, Sluijter JP, Verlaan CW, et al. Cyclooxygenase-2 inhibition increases mortality, enhances left ventricular remodeling, and impairs systolic function after myocardial infarction in the pig. *Circulation.* Jan 23 2007;115(3):326-332.

19. Steendijk P, Baan J, Jr., Van der Velde ET, et al. Effects of critical coronary stenosis on global systolic left ventricular function quantified by pressure-volume relations during dobutamine stress in the canine heart. *J Am Coll Cardiol.* Sep 1998;32(3):816-826.

20. Hausenloy DJ, Yellon DM. New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway. *Cardiovasc Res.* Feb 15 2004;61(3):448-460.

21. Dumont EA, Reutelingsperger CP, Smits JF, et al. Real-time imaging of apoptotic cell-membrane changes at the single-cell level in the beating murine heart. *Nat Med.* Dec 2001;7(12):1352-1355.

22. Eefting F, Rensing B, Wigman J, et al. Role of apoptosis in reperfusion injury. *Cardiovasc Res.* Feb 15 2004;61(3):414-426.

23. Mirotsou M, Zhang Z, Deb A, et al. Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. *Proc Natl Acad Sci U S A.* Jan 30 2007;104(5):1643-1648.

24. Dhalla NS, Elmoselhi AB, Hata T, et al. Status of myocardial antioxidants in ischemia-reperfusion injury. *Cardiovasc Res.* Aug 18 2000;47(3):446-456.

25. Redondo S, Ruiz E, Santos-Gallego CG, et al. Pioglitazone induces vascular smooth muscle cell apoptosis through a peroxisome proliferator-activated receptor-gamma, transforming growth factor-beta1, and a Smad2-dependent mechanism. *Diabetes.* Mar 2005;54(3):811-817.

26. Bolli R, Zughaib M, Li XY, et al. Recurrent ischemia in the canine heart causes recurrent bursts of free radical production that have a cumulative effect on contractile function. A pathophysiological basis for chronic myocardial "stunning". *J Clin Invest.* Aug 1995;96(2):1066-1084.

27. Dai W, Hale SL, Kloner RA. Role of a paracrine action of mesenchymal stem cells in the improvement of left ventricular function after coronary artery occlusion in rats. *Regen Med.* Jan 2007;2(1):63-68.

28. Bolli R, Becker L, Gross G, et al. Myocardial protection at a crossroads: the need for translation into clinical therapy. *Circ Res.* Jul 23 2004;95(2):125-134.

REFERENCES (INCLUDING FOR EXAMPLES 19 TO 30)

1. Le Blanc K, Pittenger M. Mesenchymal stem cells: progress toward promise. Cytotherapy. 2005;7(1):36-45.

2. Reiser J, Zhang XY, Hemenway CS, Mondal D, Pradhan L, La Russa VF. Potential of mesenchymal stem cells in gene therapy approaches for inherited and acquired diseases. Expert Opin Biol Ther. 2005;5(12):1571-1584.

3. Hui JH, Ouyang HW, Hutmacher DW, Goh JC, Lee EH. Mesenchymal stem cells in musculoskeletal tissue engineering: a review of recent advances in National University of Singapore. Ann Acad Med Singapore. 2005;34(2):206-212.

4. Caplan AI. Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. Tissue Eng. 2005;11(7-8):1198-1211.

5. Menasche P. The potential of embryonic stem cells to treat heart disease. Curr Opin Mol Ther. 2005;7(4):293-299.

6. Laflamme MA, Murry CE. Regenerating the heart. Nat Biotechnol. 2005;23(7):845-856.

7. Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem. 2006.

8. Kinnaird T, Stabile E, Burnett MS, Epstein SE. Bone-marrow-derived cells for enhancing collateral development: mechanisms, animal data, and initial clinical experiences. Circ Res. 2004;95(4):354-363.

9. Leedham SJ, Brittan M, McDonald SA, Wright NA. Intestinal stem cells. J Cell Mol Med. 2005;9(1):11-24.

10. Togel F, Hu Z, Weiss K, Isaac J, Lange C, Westenfelder C. Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. Am J Physiol Renal Physiol. 2005;289(1):F31-42.

11. Patschan D, Plotkin M, Goligorsky MS. Therapeutic use of stem and endothelial progenitor cells in acute renal injury: ca ira. Curr Opin Pharmacol. 2006;6(2):176-183.

12. Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H, Ishino K, Ishida H, Shimizu T, Kangawa K, Sano S, Okano T, Kitamura S, Mori H. Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nat Med. 2006;12(4):459-465.

13. Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F, Mu H, Melo LG, Pratt RE, Ingwall JS, Dzau VJ. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. Faseb J. 2006;20(6):661-669.

14. Gnecchi M, He H, Liang OD, Melo LG, Morello F, Mu H, Noiseux N, Zhang L, Pratt RE, Ingwall JS, Dzau VJ. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med. 2005;11(4):367-368.

15. Mayer H, Bertram H, Lindenmaier W, Korff T, Weber H, Weich H. Vascular endothelial growth factor (VEGF-A) expression in human mesenchymal stem cells: autocrine and paracrine role on osteoblastic and endothelial differentiation. J Cell Biochem. 2005;95(4):827-839.

16. Nakagami H, Maeda K, Morishita R, Iguchi S, Nishikawa T, Takami Y, Kikuchi Y, Saito Y, Tamai K, Ogihara T, Kaneda Y. Novel autologous cell therapy in ischemic limb disease through growth factor secretion by cultured adipose tissue-derived stromal cells. Arterioscler Thromb Vasc Biol. 2005;25(12):2542-2547.

17. Van Overstraeten-Schlogel N, Beguin Y, Gothot A. Role of stromal-derived factor-1 in the hematopoietic-supporting activity of human mesenchymal stem cells. Eur J Haematol. 2006.

18. Cheng L, Qasba P, Vanguri P, Thiede MA. Human mesenchymal stem cells support megakaryocyte and pro-platelet formation from CD34(+) hematopoietic progenitor cells. J Cell Physiol. 2000;184(1):58-69.

19. Lian Q, Lye E, Suan Yeo K, Khia Way Tan E, Salto-Tellez M, Liu TM, Palanisamy N, El Oakley RM, Lee EH, Lim B, Lim SK. Derivation of Clinically Compliant MSCs from CD105+, CD24- Differentiated Human ESCs. Stem Cells. 2007;25(2):425-436.

20. Sze SK, de Kleijn DP, Lai RC, Khia Way Tan E, Zhao H, Yeo KS, Low TY, Lian Q, Lee CN, Mitchell W, El Oakley RM, Lim SK. Elucidating the secretion proteome of human embryonic stem cell-derived mesenchymal stem cells. Mol Cell Proteomics. 2007;6(10):1680-1689.

21. Pan BT, Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. Cell. 1983;33(3):967-978.

22. Fevrier B, Raposo G. Exosomes: endosomal-derived vesicles shipping extracellular messages. Curr Opin Cell Biol. 2004;16(4):415-421.

23. Shen V, Kiledjian M. A view to a kill: structure of the RNA exosome. Cell. 2006;127(6):1093-1095.

24. Johnstone RM. The Jeanne Manery-Fisher Memorial Lecture 1991. Maturation of reticulocytes: formation of exosomes as a mechanism for shedding membrane proteins. Biochem Cell Biol. 1992;70(3-4):179-190.

25. Denzer K, Kleijmeer MJ, Heijnen HF, Stoorvogel W, Geuze HJ. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. J Cell Sci. 2000;113 Pt 19:3365-3374.

26. de Gassart A, Geminard C, Hoekstra D, Vidal M. Exosome secretion: the art of reutilizing nonrecycled proteins? Traffic (Copenhagen, Denmark). 2004;5(11):896-903.

27. Pelchen-Matthews A, Raposo G, Marsh M. Endosomes, exosomes and Trojan viruses. Trends Microbiol. 2004;12(7):310-316.

28. Pilzer D, Gasser O, Moskovich O, Schifferli JA, Fishelson Z. Emission of membrane vesicles: roles in complement resistance, immunity and cancer. Springer Semin Immunopathol. 2005;27(3):375-387.

29. Aoki N. Regulation and functional relevance of milk fat globules and their components in the mammary gland. Biosci Biotechnol Biochem. 2006;70(9):2019-2027.

30. Delcayre A, Le Pecq JB. Exosomes as novel therapeutic nanodevices. Curr Opin Mol Ther. 2006;8(1):31-38.

31. Johnstone RM. Exosomes biological significance: A concise review. Blood Cells Mol Dis. 2006;36(2):315-321.

32. Keller S, Sanderson MP, Stoeck A, Altevogt P. Exosomes: from biogenesis and secretion to biological function. Immunol Lett. 2006;107(2):102-108.

33. van Niel G, Porto-Carreiro I, Simoes S, Raposo G. Exosomes: a common pathway for a specialized function. J Biochem (Tokyo). 2006;140(1):13-21.

34. Hao S, Moyana T, Xiang J. Review: cancer immunotherapy by exosome-based vaccines. Cancer biotherapy & radiopharmaceuticals. 2007;22(5):692-703.

35. Subra C, Laulagnier K, Perret B, Record M. Exosome lipidomics unravels lipid sorting at the level of multivesicular bodies. Biochimie. 2007;89(2):205-212.

36. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9(6):654-659.

37. Fevrier B, Vilette D, Laude H, Raposo G. Exosomes: a bubble ride for prions? Traffic (Copenhagen, Denmark). 2005;6(1):10-17.

38. Mallegol J, van Niel G, Heyman M. Phenotypic and functional characterization of intestinal epithelial exosomes. Blood Cells Mol Dis. 2005;35(1):11-16.

39. Porto-Carreiro I, Fevrier B, Paquet S, Vilette D, Raposo G. Prions and exosomes: from PrPc trafficking to PrPsc propagation. Blood Cells Mol Dis. 2005;35(2):143-148.

40. Soderberg A, Barral AM, Soderstrom M, Sander B, Rosen A. Redox-signaling transmitted in trans to neighboring cells by melanoma-derived TNF-containing exosomes. Free Radic Biol Med. 2007;43(1):90-99.

41. Calzolari A, Raggi C, Deaglio S, Sposi NM, Stafsnes M, Fecchi K, Parolini I, Malavasi F, Peschle C, Sargiacomo M, Testa U. TfR2 localizes in lipid raft domains and is released in exosomes to activate signal transduction along the MAPK pathway. J Cell Sci. 2006;119(Pt 21):4486-4498.

42. de Gassart A, Geminard C, Fevrier B, Raposo G, Vidal M. Lipid raft-associated protein sorting in exosomes. Blood. 2003;102(13):4336-4344.

43. London E, Brown DA. Insolubility of lipids in triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts). Biochim Biophys Acta. 2000;1508(1-2):182-195.

44. Fevrier B, Vilette D, Archer F, Loew D, Faigle W, Vidal M, Laude H, Raposo G. Cells release prions in association with exosomes. Proc Natl Acad Sci U S A. 2004;101(26):9683-9688.

45. Keller S, Rupp C, Stoeck A, Runz S, Fogel M, Lugert S, Hager HD, Abdel-Bakky MS, Gutwein P, Altevogt P. CD24 is a marker of exosomes secreted into urine and amniotic fluid. Kidney Int. 2007;72(9):1095-1102.

46. Birnbaum Y, Hale SL, Kloner RA. Differences in reperfusion length following 30 minutes of ischemia in the rabbit influence infarct size, as measured by triphenyltetrazolium chloride staining. J Mol Cell Cardiol. 1997;29(2):657-666.

47. Timmers L, Sluijter JP, Verlaan CW, Steendijk P, Cramer MJ, Emons M, Strijder C, Grundeman PF, Sze SK, Hua L, Piek JJ, Borst C, Pasterkamp G, de Kleijn DP. Cyclooxygenase-2 inhibition increases mortality, enhances left ventricular remodeling, and impairs systolic function after myocardial infarction in the pig. Circulation. 2007;115(3):326-332.

48. Steendijk P, Baan J, Jr., Van der Velde ET, Baan J. Effects of critical coronary stenosis on global systolic left ventricular function quantified by pressure-volume relations during dobutamine stress in the canine heart. J Am Coll Cardiol. 1998;32(3):816-826.

REFERENCES (INCLUDING FOR EXAMPLES 31 TO 47)

Caplan, A.I., and Dennis, J.E. (2006a). Mesenchymal stem cells as trophic mediators. J Cell Biochem 98, 1076-1084.

Caplan, A.I., and Dennis, J.E. (2006b). Mesenchymal stem cells as trophic mediators. J Cell Biochem.

Fevrier, B., and Raposo, G. (2004). Exosomes: endosomal-derived vesicles shipping extracellular messages. Curr Opin Cell Biol 16, 415-421.

Gnecchi, M., He, H., Liang, O.D., Melo, L.G., Morello, F., Mu, H., Noiseux, N., Zhang, L., Pratt, R.E., Ingwall, J.S., et al. (2005). Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 11, 367-368.

Gnecchi, M., He, H., Noiseux, N., Liang, O.D., Zhang, L., Morello, F., Mu, H., Melo, L.G., Pratt, R.E., Ingwall, J.S., et al. (2006). Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. Faseb J 20, 661-669.

Keller, S., Sanderson, M.P., Stoeck, A., and Altevogt, P. (2006). Exosomes: from biogenesis and secretion to biological function. Immunol Lett 107, 102-108.

Lian, Q., Lye, E., Suan Yeo, K., Khia Way Tan, E., Salto-Tellez, M., Liu, T.M., Palanisamy, N., El Oakley, R.M., Lee, E.H., Lim, B., et al. (2007). Derivation of Clinically Compliant MSCs from CD105+, CD24- Differentiated Human ESCs. Stem Cells 25, 425-436.

Liu, C.H., and Hwang, S.M. (2005). Cytokine interactions in mesenchymal stem cells from cord blood. Cytokine 32, 270-279.

Minguell, J.J., and Erices, A. (2006). Mesenchymal stem cells and the treatment of cardiac disease. Exp Biol Med (Maywood) 231, 39-49.

Olver, C., and Vidal, M. (2007). Proteomic analysis of secreted exosomes. Sub-cellular biochemistry 43, 99-131.

Pittenger, M.F., and Martin, B.J. (2004). Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res 95, 9-20.

Saraste, A., Pulkki, K., Kallajoki, M., Henriksen, K., Parvinen, M., and Voipio-Pulkki, L.M. (1997). Apoptosis in human acute myocardial infarction. Circulation 95, 320-323.

Schafer, R., and Northoff, H. (2008). Cardioprotection and cardiac regeneration by mesenchymal stem cells. Panminerva medica 50, 31-39.

Smalheiser, N.R. (2007). Exosomal transfer of proteins and RNAs at synapses in the nervous system. Biology direct 2, 35.

Sze, S.K., de Kleijn, D.P., Lai, R.C., Khia Way Tan, E., Zhao, H., Yeo, K.S., Low, T.Y., Lian, Q., Lee, C.N., Mitchell, W., et al. (2007). Elucidating the secretion proteome of human embryonic stem cell-derived mesenchymal stem cells. Mol Cell Proteomics 6, 1680-1689.

Taylor, D.D., and Gercel-Taylor, C. (2008). MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol 110, 13-21.

Thery, C., Zitvogel, L., and Amigorena, S. (2002). Exosomes: composition, biogenesis and function. Nat Rev Immunol 2, 569-579.

Timmers, L., Lim, S.-K., Arslan, F., Armstrong, J.S., Hoefler, I.E., Doevendans, P.A., Piek, J.J., El Oakley, R.M., Choo, A., Lee, C.N., et al. (2008). Reduction of myocardial infarct size by human

mesenchymal stem cell conditioned medium. Stem Cell Research 1, 129-137.

Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J.J., and Lotvall, J.O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9, 654-659.

Zimmet, J.M., and Hare, J.M. (2005). Emerging role for bone marrow derived mesenchymal stem cells in myocardial regenerative therapy. Basic Res Cardiol 100, 471-481.

Each of the applications and patents mentioned in this document, and each document cited or referenced in each of the above applications and patents, including during the prosecution of each of the applications and patents ("application cited documents") and any manufacturer's instructions or catalogues for any products cited or mentioned in each of the applications and patents and in any of the application cited documents, are hereby incorporated herein by reference. Furthermore, all documents cited in this text, and all documents cited or referenced in documents cited in this text, and any manufacturer's instructions or catalogues for any products cited or mentioned in this text, are hereby incorporated herein by reference.

Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments and that many modifications and additions thereto may be made within the scope of the invention. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the claims. Furthermore, various combinations of the features of the following dependent claims can be made with the features of the independent claims without departing from the scope of the present invention.

CLAIMS

1. An exosome isolated from a mesenchymal stem cell and having a size of between 50 nm and 100 nm as determined by electron microscopy, the exosome comprising at least one biological property of a mesenchymal stem cell or a biological activity of a mesenchymal stem cell conditioned medium (MSC-CM), preferably cardioprotection.

2. An exosome according to Claim 1, which is capable of reducing infarct size as assayed in a mouse or pig model of myocardial ischemia and reperfusion injury, or which is capable of reducing oxidative stress as assayed in an *in vitro* assay of hydrogen peroxide (H_2O_2)-induced cell death.

3. An exosome according to Claim 1 or 2, in which the exosome comprises at least 70% of proteins in an mesenchymal stem cell conditioned medium (MSC-CM), preferably selected from the list shown in Table D1 or E2 or are gene products of the genes shown in Table D2 or comprising one or more miRNAs as shown in Table E3.

4. An exosome according to Claim 1, 2 or 3, in which the exosome comprises: (a) a complex of molecular weight >100 kDa, preferably comprising proteins of <100 kDa; (b) a complex of molecular weight > 300 kDa, preferably comprising proteins of < 300 kDa; or (c) a complex of molecular weight > 1000 kDa.

5. An exosome according to any preceding claim, in which the exosome has a size of between 50 nm and 100 nm preferably as determined by filtration against a 0.2μM filter and concentration against a membrane with a molecular weight cut-off of 10 kDa, or a hydrodynamic radius of below 100 nm, preferably between about 30 nm and about 70 nm, between about 40 nm and about 60 nm, preferably between about 45 nm and about 55 nm, preferably about 50 nm, preferably as determined by laser diffraction or dynamic light scattering.

6. An exosome according to any preceding claim, in which the exosome comprises a lipid selected from the group consisting of: phospholipid, phosphatidyl serine, phosphatidyl inositol, phosphatidyl choline, shingomyelin, ceramides, glycolipid, cerebroside, steroids, cholesterol, preferably in which the cholesterol-phospholipid ratio is greater than 0.3-0.4 (mol/mol).

7. An exosome according to any preceding claim, in which the exosome comprises a lipid raft, or in which the exosome is insoluble in non-ionic detergent, preferably Triton-X100 or in which the exosome is such that proteins of the molecular weights specified in Claim 4 substantially remain in the complexes of the molecular weights specified in those claims, when the exosome is treated with a non-ionic detergent, or in which the exosome is sensitive to cyclodextrin, preferably 20 mM cyclodextrin, such that preferably treatment with cyclodextrin causes substantial dissolution of the complexes specified in Claim 4.

8. An exosome according to any preceding claim, in which the exosome comprises ribonucleic acid (RNA), preferably in which the exosome has an absorbance ratio of 1.9 (260:280 nm), or in which the exosome comprises a surface antigen selected from the group consisting of: CD9, CD109 and thy-1.

9. A method of producing a exosome according to any preceding claim, the method comprising isolating the exosome from a mesenchymal stem cell conditioned medium (MSC-CM), the method optionally comprising separating the exosome from other components based on molecular weight, size, shape, composition or biological activity, preferably in which the weight is selected from the weights set out in Claim 4 or in which the size is selected from the sizes set out in Claim 5 or in which the composition is selected from the compositions set out in Claim 6 or in which the biological activity is selected from the biological activities set out in any of Claim 1 or 2.

10. A method of producing an exosome according to any of Claims 1 to 8, the method comprising:

(a) obtaining a mesenchymal stem cell conditioned medium (MSC-CM);

(b) concentrating the mesenchymal stem cell conditioned medium, preferably by ultrafiltration over a >1000 kDa membrane;

(c) subjecting the concentrated mesenchymal stem cell conditioned medium to size exclusion chromatography, preferably using a a TSK Guard column SWXL, 6 x 40 mm or a TSK gel G4000 SWXL, 7.8 x 300 mm column; and

(d) selecting UV absorbant fractions, preferably at 220 nm, that exhibit dynamic light scattering, preferably detected by a quasi-elastic light scattering (QELS);

in which step (d) preferably comprises collecting fractions which elute with a retention time of 11-13 minutes, preferably 12 minutes.

11. A pharmaceutical composition comprising an exosome according to any of Claims 1 to 8 together with a pharmaceutically acceptable excipient, diluent or carrier.

12. An exosome according to any of Claims 1 to 8 or a pharmaceutical composition according to Claim 11 for use in a method of treating a disease.

13. Use of an exosome according to any of Claims 1 to 8 for the preparation of a pharmaceutical composition for the treatment of a disease in an individual.

14. An exosome or a pharmaceutical composition according to Claim 12, or a use according to Claim 13 in which the disease is selected from the group consisting of: cardiac failure, bone marrow disease, skin disease, burns and degenerative diseases, diabetes, Alzheimer's disease, Parkinson's disease, cancer, myocardial infarction, a cutaneous wound, a dermatologic disorder, a dermatological lesion, dermatitis, psoriasis, condyloma, verruca, hemangioma, keloid, skin cancer, atopic dermatitis, Behcet disease, chronic granulomatous disease, cutaneous T cell lymphoma, ulceration, a pathological condition characterised by initial injury inducing inflammation and immune dysregulation leading to chronic tissue remodeling including fibrosis and loss of function, renal ischemic injury, cystic fibrosis, sinusitis and rhinitis or an orthopaedic disease.

15. An exosome according to Claim 12, or a use according to Claim 13 in which the method of treatment comprises use of an exosome to aid wound healing, scar reduction, bone formation, a bone graft or bone marrow transplantation in an individual, or (i) in the regulation of a pathway selected from any one or more of the following: cytoskeletal regulation by Rho GTPase, cell cycle, integrin signalling pathway, Inflammation mediated by chemokine & cytokine signaling pathway, FGF signaling pathway, EGF receptor signaling pathway, angiogenesis, plasminogen activating cascade, blood coagulation, glycolysis, ubiquitin proteasome pathway, de novo purine biosynthesis, TCA cycle, phenylalanine biosynthesis, heme biosynthesis; (ii) in the regulation of processes including any one or more of the following: cell structure and motility, cell structure, cell communication, cell motility, cell adhesion, endocytosis, mitosis, exocytosis, cytokinesis, cell cycle, immunity and defense, cytokine/chemokine mediated immunity, macrophage-

mediated immunity, granulocyte-mediated immunity, ligand-mediated signaling, cytokine and chemokine mediated signaling pathway, signal transduction, extracellular matrix protein-mediated signaling, growth factor homeostasis, receptor protein tyrosine kinase signaling pathway, cell adhesion-mediated signaling, cell surface receptor mediated signal transduction, JAK-STAT cascade, antioxidation and free radical removal, homeostasis, stress response, blood clotting, developmental processes, mesoderm development, skeletal development, angiogenesis, muscle development, muscle contraction, protein metabolism and modification, proteolysis, protein folding, protein complex assembly, amino acid activation, intracellular protein traffic, other protein targeting and localization, amino acid metabolism, protein biosynthesis, protein disulfide-isomerase reaction, carbohydrate metabolism, glycolysis, pentose-phosphate shunt, other polysaccharide metabolism, purine metabolism, regulation of phosphate metabolism, vitamin metabolism, amino acid biosynthesis, pre-mRNA processing, translational regulation, mRNA splicing; or (iii) in the supply of functions including any one or more of the following: signaling molecule, chemokine, growth factor, cytokine, interleukin, other cytokine, extracellular matrix, extracellular matrix structural protein, other extracellular matrix, extracellular matrix glycoprotein, protease, metalloprotease, other proteases, protease inhibitor, metalloprotease inhibitor, serine protease inhibitor, oxidoreductase, dehydrogenase, peroxidase, chaperone, chaperonin, Hsp 70 family chaperone, other chaperones, synthetase, synthase and synthetase, select calcium binding protein, aminoacyl-tRNA synthetase, lyase, isomerase, other isomerase, ATP synthase, hydratase, transaminase, other lyase, other enzyme regulator, select regulatory molecule, actin binding cytoskeletal protein, cytoskeletal protein, non-motor actin binding protein, actin and actin related protein, annexin, tubulin, cell adhesion molecule, actin binding motor protein, intermediate filament, ribonucleoprotein, ribosomal protein, translation factor, other RNA-binding protein, histone, calmodulin related protein, vesicle coat protein.

16. A delivery system for delivering an exosome according to any of Claims 1 to 8, comprising a source of exosome together with a dispenser operable to deliver the exosome to a target.

17. A delivery system according to Claim 16 for use in a method of delivering an exosome to a target.