METHODS OF DETECTING THERAPEUTIC EXOSOMES

FIELD

The present invention relates to the fields of medicine, cell biology, molecular biology and genetics. This invention relates to the field of medicine.

BACKGROUND

Exosomes were once thought to be "trash bags" for cells to discard unwanted proteins[1]. However, exosomes are increasingly viewed as having important physiological function particularly in cellular communication.

Exosomes are bi-lipid membrane vesicles of 50-100 ηm that are secreted by many cell types[2]. They belong to a class of secreted cellular products known as microparticles which broadly encompasses all secreted membrane vesicles. Other than exosomes, microparticles include microvesicles (100-1000 ηm), ectosomes (50-200 ηm), membrane particles (50-80 ηm), exosome-like vesicles (20-50 ηm) and apoptotic vesicles (50-500 ηm). The major distinguishing parameter for these different classes of microparticles is their size and the best defined class is the exosomes.

Exosomes have a density in sucrose of 1.10 to 1.19 g/ml, sedimented at 100,000 g, has a cholesterol-rich lipid membrane containing sphingomyelin, ceramide, lipid rafts and exposed phosphatidylserine. The process of exosome biogenesis is complex and involves complex intracellular membrane trafficking and cargo sorting through the biosynthetic and endocytotic pathways. As evidence of this complex biogenesis, the hallmark features of exosomes are markers of the endoplasmic reticulum and the endosomes such as Alix, Tsg101, Rab proteins, etc. Exosomes are stored in multivesicular bodies prior to release via fusion of the multivesicular bodies (MVBs) with the plasma membrane.

Exosomes have been shown to mediate intercellular communication particularly in immune or tumor cells[3-8]. Recently we extended this function to include tissue repair when we reported that exosomes secreted by human ESC-derived MSCs reduced infarct size by about 50% in a mouse model of myocardial ischemia reperfusion (MI/R) injury[9,10]. These exosomes were purified as a population of homogenously sized microparticles of about 50-100 ηm in diameter by size exclusion on HPLC and they carry both protein and RNA load[9,11,12].

However, the mechanism/s underlying this cardioprotective effect by MSC exosomes has yet to be elucidated. Part of the reason could be attributed to a deficiency in our understanding of the biological potency of exosomes in general. Despite extensive proteomic and RNA profiling of exosomes from various cell types and biological fluids, the biological potency of the proteins and RNAs in exosomes remains largely uninvestigated. Most studies to date on the biological potency are limited to the immune responses that are elicited by exosomes from immune cells, particularly the dendritic cells[2] but the molecular or biochemical basis of these biological responses have yet to be elucidated.

SUMMARY

To address this deficiency, we performed a comprehensive proteomic profiling of HPLC-purified exosome to first identify proteins present in these exosomes and then using these proteins to predict the types of biological activity or potential in the exosomes. This was then validated by either biochemical or cellular assays.

A total of 866 unique gene products were detected and they could be functionally clustered into 32 over-represented biological processes indicating that exosomes have the potential to exert a wide spectrum of biochemical or cellular effects. To evaluate this potential, we selected proteins for which assays to assess either their biochemical and/or cellular activities are available and that together, would demonstrate the wide spectrum of biochemical and cellular potential in exosomes, and provide candidate molecular mechanisms for the cardioprotective properties of MSC exosomes.

The proteins investigated here include glycolytic enzymes for the breakdown of glucose to generate ATP and NADH, PFKB3 that increases glycolysis, CD73 that hydrolyses AMP to adenosine capable of activating signaling cascades through adenosine receptors, CD59 that inhibits the formation of membrane attack complex (MAC) and 20S proteasome.

According to a 1st aspect of the present invention, we provide a method of detecting a therapeutic exosome. The method may comprise detecting an activity of an exosome. The method may comprise immunodulatory activity. It may comprise complement inhibition activity. It may comprise proteasome activity. It may comprise glycolytic enzyme activity. It may comprise anti-oxidative activity. It may comprise extracellular matrix (ECM) modifying activity. It may comprise NT5E (CD73) ecto-5'-ectonucleotidase activity. It may comprise ion

homeostasis activity. It may comprise chaperone activity. It may comprise any one or more of the above.

The activity may comprise immunodulatory activity. The immunodulatory activity may be detected by detecting activity of a protein set out in Table D10.

The activity may comprise complement inhibition activity. The complement inhibition activity may be detected by detecting activity of a protein set out in Table D1.

The activity may comprise proteasome activity. The proteasome activity may be detected by detecting activity of one or more, preferably all, proteins set out in Table D2.

The activity may comprise glycolytic enzyme activity. The glycolytic enzyme activity may be detected by detecting activity of one or more, preferably all, proteins set out in Table D3.

The activity may comprise anti-oxidative activity. The anti-oxidative activity may be detected by detecting activity of one or more, preferably all, proteins set out in Table D4.

The activity may comprise extracellular matrix (ECM) modifying activity. The extracellular matrix (ECM) modifying activity may be detected by detecting activity of one or more, preferably all, proteins set out in Table D5.

The activity may comprise NT5E (CD73) ecto-5'-ectonucleotidase activity. The NT5E (CD73) ecto-5'-ectonucleotidase activity may be detected by detecting activity of one or more, preferably all, proteins set out in Table D6.

The activity may comprise ion homeostasis activity. The ion homeostasis activity may be detected by detecting activity of one or more, preferably all, proteins set out in Table D7.

The activity may comprise chaperone activity. The chaperone activity may be detected by detecting activity of one or more, preferably all, proteins set out in Table D8.

The activity may comprise immunomodulatory activity. The immunomodulatory activity may be detected by detecting activity of one or more, preferably all, proteins set out in Table D10.

If an activity is detected, the exosome may be likely to be capable of inhibiting complement mediated cell lysis. It may also, alternatively or in addition, be capable of

reducing infarct size for example as assayed in a mouse or pig model of myocardial ischemia and reperfusion injury, or which is capable of reducing oxidative stress for example as assayed in an *in vitro* assay of hydrogen peroxide (H_2O_2)-induced cell death. It may have both the above activities.

If an activity is detected, the exosome may be likely to comprise a therapeutic exosome having therapeutic activity.

The therapeutic activity may comprise cardioprotective activity.

The therapeutic activity may be against one or more diseases 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, 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.

There is provided, according to a 2nd aspect of the present invention, a method comprising the steps of: (a) isolating an exosome from a mesenchymal stem cell conditioned medium (MSC-CM), optionally comprising separating the exosome from other components based on molecular weight, size, shape, composition or biological activity, and (b) detecting an activity as set out above in the isolated exosome, preferably by detecting the activity of one or more proteins as set out above.

We provide, according to a 3rd aspect of the present invention, a method of producing an exosome, the method comprising: (a) obtaining a mesenchymal stem cell conditioned medium (MSC-CM); (b) concentrating the mesenchymal stem cell conditioned medium, for example by ultrafiltration over a >1000 kDa membrane; (c) subjecting the concentrated mesenchymal stem cell conditioned medium to size exclusion chromatography, such as using a a TSK Guard column SWXL, 6 x 40 mm or a TSK gel G4000 SWXL, 7.8 x 300 mm column; (d) selecting UV absorbant fractions, for example, at 220 nm, that exhibit dynamic light scattering, such as detected by a quasi-elastic light scattering (QELS); in which step (d) for example comprises collecting fractions which elute with a retention time of 11-13 minutes,

such as 12 minutes; and detecting an activity as set out above in the exosome, preferably by detecting the activity of one or more proteins as set out above.

As a 4th aspect of the present invention, there is provided a method of detecting whether an exosome comprises therapeutic activity, the method comprising detecting an enzymatic activity as set out above, in which if the enzymatic activity is detected, then the exosome comprises therapeutic activity.

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, *In Situ Hybridization: Principles and Practice*; Oxford University Press; M. J. Gait (Editor), 1984, *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. Handbook of Drug Screening, edited by Ramakrishna Seethala, Prabhavathi B. Fernandes (2001, New York, NY, Marcel Dekker, ISBN 0-8247-0562-9); 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 is a diagram showing the intersection of the 739 proteins previously identified in MSC conditioned medium versus the 866 proteins identified in purified exosomes.

Figure 2 is a diagram showing proteomic analysis of exosome proteins. The 866 gene products in exosomes were functionally clustered into 32 over-represented and three under-represented biological processes ($p<0.001$). The 421 proteins found in the conditioned medium (CM) but not in the exosome were functionally clustered into 11 over-represented and 2 under-represented biological processes. Black bars represent processes for gene products in the exosomes and white bars represent processes for gene products in the CM but not exosomes.

Figures 3A to 3D are diagrams showing that exosomes regulate glycolysis.

Figure 3A is a schematic diagram of glycolysis.

Figure 3B is a Western blot analysis of conditioned medium (CM) and exosome (Exo) for geraldehyde phosphate dehydrogenase (GAPDH), phosphoclycerate kinase (PGK), pyruvate kinase m2 isoform (PKm_2) and pPFKFB3.

Figure 3C is a Enzymatic activities of GAPDH, PGK and PKm_2 in MSC exosome were determined by the production of ATP or pyruvate using commercially available assay kit. One unit (U) enzyme activity is defined as the activity to generate 1 μmole product per minute at 37°C.

Figure 3D. Effect of exosome on ATP production in oligomycin-treated cells. H9C2 cardiomyocytes were washed twice with Tyrode's buffer and then incubated in Tyrode's buffer containing 20 μmol of a mitochondrial inhibitor, oligomycin, 6 mmol glucose, and with or without 0.1 μg/ml exosomes for 15, 30 and 60 minutes. Cellular ATP concentration was measured using ATPlite 1step luminescence ATP detection assay system and normalized to that of sample without exosomes at 15 minutes. $*p=0.0173$, $**p=0.0090$

Figures 4A and 4B are diagrams showing 20S proteasome in exosome.

Figure 4A. Western blot analysis of MSC conditioned medium (CM) and exosome (Exo) using an antibody specific for PMSA 1-7 peptides.

Figure 4B. Proteasome activity in MSC exosome was determined using a commercially available proteasome activity assay kit (Millipore). Proteasome activity was measured by the rate of degradation of a fluorogenic peptide in the absence or presence of

lactacystin, a proteasome inhibitor. One unit (U) enzyme activity is defined as the activity to generate 1 μmole product per minute at 37°C. *p=0.00023

Figures 5A to 5C are diagrams showing exosome phosphorylated ERK and AKT via NT5E (ecto-5'-ectonucleotidase CD73).

Figure 5A. Western blot analysis of MSC CM and exosome for CD73 using a specific antibody.

Figure 5B. CD73 activity in conditioned medium (CM) and exosomes (Exo) was measured by the production of phosphate ion from the hydrolysis of AMP.

Figure 5C. H9C2 cells were serum starved overnight and then incubated with medium with or without 1mM theophylline for one hour. The cells were then exposed for 5 min to medium that had been pre-incubated for 30 minutes with 50 μM AMP, 0.1μg/mL exosome or AMP and exosome. The cells were then harvested and lysed. 10 μg total proteins were immunoblotted using 1:2000 dilution of rabbit anti-pERK 1/2, 1:2000 dilution of rabbit anti ERK1/2, 1:500 dilution rabbit anti-pAKT or 1:500 dilution of rabbit anti AKT.

Figures 6A to 6B are diagrams showing exosome inhibited the formation of membrane attack complex (MAC).

Figure 6A. Western blot analysis of MSC conditioned medium (CM) and exosome (Exo) using a CD59-specific antibody.

Figure 6B. SRBCs were washed and then re-suspended in PBS with C5b6 and C7 in the presence or absence of exosome. The mixture was incubated at 37°C for 15 min before C8 and C9 were added with or without a blocking CD59 antibody for additional 30 min incubation. The cells were centrifuged and the amount of hemoglobin released by the lysed SRBC in the supernatant was measured by absorbance at 415 nm. The positive control (total hemolysis) was supernatant from cells completely lysed with Triton X-100. The negative control is the sample without addition of complement components. The absorbance value of positive control was normalized to 100%*p=2.8E-06, **p=3.51E-08.

Figure 7 is a diagram showing that MSC exosome has TLR ligands.

Figure 8 is a diagram showing that MSC exosome does not activate TLR2.

Figure 9 is a diagram showing that MSC exosome activates TLR4.

Figure 10 is a diagram showing that MSC exosome induce production of pro- and anti-inflammatory cytokines by THP-1 cells.

Figure 11 is a diagram showing that inducing differentiation of CD4+ mouse T cells into either Treg (a) or Th17 cells (b).

Figure 12A and Figure 12B are diagrams showing modulating adaptive immunity via innate immunity.

Figure 13 is a diagram showing a concanavalin A-activated lymphocyte proliferation assay for exosomes.

DETAILED DESCRIPTION

Mammalian cells secrete exosomes that contain proteins and RNAs. Hypothetically, these exosomes could be harvested and purified from healthy cells to be used to replenish and bolster the cellular resources in diseased or distressed cells, and enable these cells to initiate recovery and repair. For example, we have demonstrated that exosomes from human embryonic stem cell-derived mesenchymal stem cells could rescue cardiomyocytes from cell death after acute myocardial ischemia-reperfusion injury. Here we describe the biochemical properties of these exosomes. These properties could be used to identify, select or assess exosome preparation for therapeutic potential in treating diseased or distressed cells.

Cells in distress as a consequence of injury or disease often share similar cellular dysfunctions. They have increased mitochondrial membrane permeability resulting in reduced ATP and NADH production, increased ROS level and loss in ion homeostasis including Na, Ca, K and Cl. There are also accumulation of denatured proteins leading to ER stress and formation of inclusion bodies and excessive innate immune response. To begin the process of recovery and repair, cells must first correct these cellular dysfunctions. Since exosomes are secreted phospholipid vesicles containing proteins and RNAs, they could potentially deliver enzymes and RNA that could rapidly initiate the necessary cellular activities to correct these cellular dysfunctions.

Here we describe the biochemical properties of therapeutic exosomes. These biochemical properties include

a. Inhibition of complement activity (Table D1 below)

b. proteasome activity to degrade denature proteins (Table D2 below)

c. glycolytic enzymes to generate ATP and NADH (Table D3below)

d. pentose phosphate pathway enzymes to generate NADPH

e. anti-oxidative activity (Table D4 below)

f. MMP/TIMP activity (Table D5 below)

g. CD73 ecto-5'-ectonucleotidase activity to activate survival signaling pathway (Table D6 below)

h. ATPase membrane channels or transporters to restore ion homeostasis e.g. calcium, sodium, proton, potassium and chloride channels (Table D7 below)

i. chaperone activity to maintain protein structural integrity (Table D8 below)

Potentially therapeutic exoosmes should not only have the proteins for these biochemical activies, the proteins must be enzymatically active. The enzymatic activity of these proteins could therefore be surrogate markers for the therapeutic efficacy of an exosome preparation.

We therefore provide a number of biochemical assays to define, identify and assess therapeutic exosomes.

ASSAYS FOR THERAPEUTIC EXOSOMES

We describe biological properties, in particular biochemical properties of a particle which is derivable from a mesenchymal stem cell (MSC), for example an exosome.

According to the methods and compositions described here, an exosome which comprises one or more of: (a) complement inhibition activity; (b) proteasome activity; (c) glycolytic enzyme activity; (d) anti-oxidative activity; (e) extracellular matrix (ECM) modifying activity; (f) NT5E (CD73) ecto-5'-ectonucleotidase activity; (g) ion homeostasis activity; (h) chaperone activity, may comprise therapeutic activity; and (i) immunodulatory activity.

An exosome comprising such properties may be therapeutic in nature against a number of diseases, as described below, in other words be a therapeutic exosome. Where the term "exosome" is used in this document, this should be understood to include "therapeutic exosome" as well, where the context permits.

The exosome may be derivable from the MSC by any of several means, for example by secretion, budding or dispersal from the MSC. For example, the exosome may be produced, exuded, emitted or shed from the MSC. Where the MSC is in cell culture, the exosome may be secreted into the cell culture medium.

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

The exosomes may comprise vesicles or a flattened sphere limited by a lipid bilayer. The exosomes may comprise diameters of 40-100 nm. The exosomes may be formed by inward budding of the endosomal membrane. The exosomes may have a density of ~1.13 – 1.19 g/ml and may float on sucrose gradients. The exosomes may be enriched in cholesterol and sphingomyelin, and lipid raft markers such as GM1, GM3, flotillin and the src protein kinase Lyn. The exosomes 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.

We provide a exosomes which comprises one or more genes found in MSCs or medium which is conditioned by culture of MSCs, and which comprises therapeutic activity. Such therapeutic exosomes may be identified by detecting exosomes which display one or more activities selected from the group consisting of: (a) complement inhibition activity; (b) proteasome activity; (c) glycolytic enzyme activity; (d) anti-oxidative activity; (e) extracellular matrix (ECM) modifying activity; (f) NT5E (CD73) ecto-5'-ectonucleotidase activity; (g) ion homeostasis activity; (h) chaperone activity; and (i) immunodulatory activity.

The exosomes may comprise molecules secreted by the MSC. Such an exosome, 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 exosomes 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 exosomes may comprise one or more tetraspanins. The exosomes may comprise mRNA and/or microRNA.

The term "particle", which includes an exosome, 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.

Where the term "particle" is used in this document, it should be understood as including (and where the context permits) synonymous with, an exosome.

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

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

Media conditioned by MSCs (such as mesenchymal stem cell conditioned media or MSC-CM, as described below) are known to comprise biological activities of MSC and are capable of substituting for the MSCs themselves - see for example WO 2009/105044. 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 exosome may comprise one or more biological properties or activities of a mesenchymal stem cell conditioned medium (MSC-CM).

Therapeutic exosomes described here may be isolated from Mesenchymal Stem Cell Conditioned Medium (MSC-CM) and tested for the presence of one or more of the following

activities: (a) complement inhibition activity; (b) proteasome activity; (c) glycolytic enzyme activity; (d) anti-oxidative activity; (e) extracellular matrix (ECM) modifying activity; (f) NT5E (CD73) ecto-5'-ectonucleotidase activity; (g) ion homeostasis activity; (h) chaperone activity; and (i) immunodulatory activity.

DETECTION OF ACTIVITIES

Each of the above activities may be detected in therapeutic exosomes by a number of ways, known in the art. For example, the relevant activity may be detected by detection of the presence of the protein or polypeptide responsible for the activity in the therapeutic exosome. This may be achieved for example by use of cognate antibodies and detection of binding between the antibody and the protein, by immunoassays and Western blots, etc as known in the art.

In general, 1 unit (U) of enzyme activity is defined as the activity required for the production of 1 μmole of product per minute.

Western Blot Protocol for Detection of Activities

An example protocol using Western blot hybridization to detect activity via proteins or polypeptides is set out below.

12 μg exosomes were separated on 4-12% SDS-polyacrylamide gels and electroblotted onto a nitrocellulose membrane. The membrane was transferred to the membrane holder of SNAP i.d. system (Millipore, Billerica, MA), blocked and incubated with primary anti-human antibodies, which included mouse anti-GAPDH (1:100 dilution), mouse anti-PGK (1:60), mouse anti-PGD (1:60), rabbit anti-PFKFB3 (1:60), mouse anti-pyruvate kinase (PK, 1:200), mouse anti- 20S proteasome α1-7 (1:200), mouse anti-CD73 (1:60) and mouse anti-CD59 (1:200) . The blot was then incubated with a horseradish peroxidase-coupled secondary antibody. The secondary antibody used was goat anti-mouse IgG (1:1250) or donkey anti-rabbit IgG (1:1250). All antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA except mouse anti-PK which was from Abcam Inc., Cambridge, MA. The blot was then incubated with HRP-enhanced Chemiluminescent substrate (Thermo Fisher Scientific Inc., Waltham, MA) and then exposed to X-ray film.

Liquid Chromatography-Mass Spectrometry (LC MS) or Mass Spectrometry (MS)

Other methods such as mass spectrometry may also be used to detect presence of the protein or polypeptide in the exosome. LC MS is described in detail in the document "Basics

of LC/MS Primer" (Agilent Technologies) at
http://www.chem.agilent.com/Library/Support/Documents/a05296.pdf

An example protocol using liquid chromatography-mass spectrometry (LC MS) or mass spectrometry (MS) is set out below.

Proteins in two ml of dialyzed exosomes were reduced, alkylated and tryptic digested as described (20). The samples were 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 were then dried to about 10% of their initial volumes by removing organic solvent in a vacuum centrifuge. To reduce sample complexity, offline peptide fractionation was carried out with a HPLC system (Shimadzu, Japan) through a Polysulfoethyl SCX column (200 mm x 4.6 mm) (PolyLC, USA). Mobile phase A (5 mM KH4PO4 + 30% acetonitrile) and mobile phase B (5 mM KH4PO4 + 30% acetonitrile + 350 mM KCl) at 1 ml/min. Eight fractions were collected and dried with a vacuum centrifuge. Fractionated samples were loaded into the auto sampler of a Shimadzu micro HPLC system coupled online to a LTQ-FT Ultra linear ion trap mass spectrometer (Thermo Electron, Bremem, Germany) fitted with a nanospray source. Injected peptides were trapped and desalted in a Zorvax 300SB-C18 enrichment column (5 mm x03 mm, Agilent Technologies, Germany) and eluted into a nano-bored C18 packed column (75 μm x 100 Å, Michrom Bioresources, Auburn, CA). A 90 l/min with a splitter to anmminute gradient at a constant flow rate of 20 effective flow rate of 200 ηl/min was used to elute the peptides into the mass spectrometer. The LTQ was 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 were combined into a single mascot generic file by a home-written program. Protein identification was achieved by searching the combined data against the IPI human protein database (version 3.34; 69,164 sequences, 29,064,825 residues) via an in-house Mascot server (Version 2.2.04, Matrix Science, UK). The search parameters were: a maximum of 2 missed cleavages using trypsin; fixed modification was carbaminomethylation of cysteine and variable modification was oxidation of methionine. The mass tolerances were set to 10 ppm and 0.8 Da for peptide precursor and fragment ions respectively. Protein identification was accepted as true positive if two different peptides were found to have scores greater than the homology scores.

COMPLEMENT INHIBITION ACTIVITY

Complement inhibition activity may be detected in therapeutic exosomes by detection of any one or more of the proteins or their activities, as set out in Table D1, thereby identifying them as therapeutic exosomes.

Table D1

Protein	GenBank Accession Number/ NCBI Reference Sequence (RefSeq)	Synonyms
CD59	NM_203329.2, NP_976074.1	

Table D1. Complement Inhibition Activity

Alternatively, or in addition, complement-mediated cell lysis activity may be detected by conducting the assay set out below.

Complement-mediated Cell Lysis Assay

Briefly, sheep red blood cells (SRBCs) were purchased (Innovative Research, Southfield, MI) and washed three times with phosphate buffered saline (PBS) before resuspending at 1×108 cell/ml PBS. Purified complement components, C5b6, C8 and C9 were purchased from Calbiochem (San Diego, CA), and C7 from Sigma-Aldrich (St Louis, MO). Assembly of intact C5b-9 on SRBCs was initiated by a 15 min incubation (37 °C) with 1ml each of C5b6 (0.1μg/ml) and C7 (0.4μg/ml) in the presence or absence of exosomes at a final concentration of 0.1 μg/ml. The SRBCs were then washed and incubated with 1ml each of C8 (0.4μg/ml,) plus C9 (0.4μg/ml) with or without a blocking CD59 antibody at a final concentration of 0.05 μg/ml for an additional 30 min. After that the SRBCs were centrifuged and the amount of hemoglobin released by the lysed SRBCs in the supernatant was measured by absorbance at 415 nm. Total (100%) hemolysis was obtained by treating the cells with 1% (w/v) Triton X-100.

PROTEASOME ACTIVITY

Proteasome activity may be detected in therapeutic exosomes by detection of any one or more of the proteins or their activities, as set out in Table D2, thereby identifying them as therapeutic exosomes.

Table D2

Protein	GenBank Accession Number/ Ref seq	Synonyms

PSMA1	X61969.1, CAA43961.1	
PSMA2	D00760.1, BAA00657.1	
PSMA3	NM_002788.2, NP_002779.1	
PSMA4	BC005361.1, AAH05361.1	
PSMA5	X61970.1, CAA43962.1	
PSMA6	X59417.1, CAA42052.1	
PSMA7	AF022815.1, AAB81515.1	
PSMB1	D00761.1, BAA00658.1	
PSMB2	D26599.1, BAA05646.1	
PSMB3	BC013008.2, AAH13008.1	
PSMB4	D26600.1, BAA05647.1	
PSMB5	D29011.1, BAA06097.1	
PSMB6	BC000835.2, AAH00835.1	
PSMB7	AJ420455.1	
20S core particle		
PSMB8	NM_004159.4, NP_004150.1	
PSMB9	NM_148954.2, NP_683756.1	
PSMB10	Y13640.1, CAA73982.1	

Table D2. Proteasome Activity

Alternatively, or in addition, proteasome activity may be detected by conducting the assay set out below.

20S Proteasome Assay

The proteasome activity was measured using a 20S proteasome activity assay kit (Millipore) based on detection of the fluorophore 7-Amino-4 methylcoumarin (AMC) after cleavage from the labeled substrate LLVY-AMC by 20S proteasome in the presence or absence of lactacystin, a specific 20S proteasome inhibitor. Briefly, 4 μg of exosome was incubated with a reaction buffer containing LLVY-AMC in the presence or absence of 25μM lactacystin. The samples and AMC standards were incubated at 37 °C and fluorescense intensity at Ex/Em= 380/460 nm was monitored for 2 hours.

Unit Activity Definition

1 unit enzyme activity of 20S core particle is defined as the activity required for the production of 1 μmole of product per minute.

Exosomes are found to contain 5.0 μU 20S core particle activity / μg protein.

GLYCOLYTIC ENZYME ACTIVITY

Glycolytic enzyme activity may be detected in exosomes by detection of any one or more of the proteins or their activities, as set out in Table D3, thereby identifying them as therapeutic exosomes.

Table D3

Protein	**GenBank Accession Number/ NCBI Reference Sequence (RefSeq)**	**Synonyms**
GAPDH	AF261085.1, AAF99678.1	
PGK	L00159.1, AAA60078.1	
PGM1	BC019920.1, AAH19920.1	
Enolase2	BC022545.1, AAH22545.1	
PKm2	M23725.1, AAA36449.1	
PFKFB3	CAH73611	
PFKFB3 - phosphorylated form		
ATP *		
NADH *		

Table D3. Glycolytic Enzyme Activity

* It is understood that ATP and NADH are not proteins. They may however be detected in order to detect glycolytic enzyme activity in exosomes and accordingly may be used to establish whether an exosome is a therapeutic exosome. Similarly, detection of ATP and NADH roduction (or both) in a cell may be used (see above).

Accordingly, it will be understood in the context of this document, that the term "protein" or "polypeptide" may encompass small molecules such as ATP and NADH. No adverse significance should be attached to such a reference to detection of an entity under a table header "protein" in such a table (or elsewhere in this document), where the entity is not a protein but is in fact some other type of entity, e.g., a small molecule. Accordingly, some table headings and other references in this document (e.g., Table D3) may for the sake of convenience refer to "proteins" but may include references to ATP, NADH, etc (where these are not in fact proteins). These may be detected, as noted above, for determination of therapeutic exosomes.

Furthermore, as glycolytic enzyme activity may be transferred into cells exposed to the exosomes, resulting in increased ATP and NADH production, detection of ATP or NADH

production (or both) by the cell may be used as a means of detecting glycolytic enzyme activity in a produced exosome, thereby identifying it as a therapeutic exosome.

Pyruvate Kinase Assay

5 μg of exosome (in 12 μl) was lysed using a cell extraction kit (Biovision, Mountain view, CA). The lysed exosome extract was incubated with 50 μl of reaction mix from a commercially available PK assay kit (Biovision). In this assay, pyruvate produced by PK was oxidized by pyruvate oxidase to produce fluorescences (Ex/Em= 535/587 nm). The increase in fluorescence intensity is therefore proportional to the amount of pyruvate produced.

GAPDH and PGK Assay

GAPDH and PGK activity were measured based on their downstream product, ATP in the glycolysis reaction using 2 commercially available kits, KDalert GAPDH assay kit (Ambion Inc., Austin, TX) and ApoSENSOR ADP/ATP ratio assay kit (Biovision). Briefly, exosomes were lysed using a cell extraction kit (Biovision). To measure GAPDH activity, 10 μg of lysed exosomes was added to KDalert reaction buffer containing D-glyceraldehyde-3-phosphate, NAD+ and Pi to form 1, 3-bisphosphoglycerate + NADH+ H+. 250 U/ml of PGK and 60 μM ADP were then added to convert 1, 3-bisphosphoglycerate and ADP to 3-phosphoglycerate and ATP. The amount of ATP produced which was proportional to GAPDH activity, was then measured using ATP luciferase assay. To measure PGK activity, 10 μg of lysed exosomes was added to the 1, 3-bisphosphoglycerate produced from the above assay. ADP was added to allow the formation of ATP. ATP amount which is proportional to PGK activity was then quantified using ATP luciferase assay.

Glycolysis Cell Based Assay

H9C2 cardiomyocytes were plated onto a 96 well plate (poly-lysine coated) at 30,000 cells per well. After 5 hours, the cells were washed twice with Tyrode's buffer before incubating in Tyrode's buffer containing 20 μmol oligomycin (Sigma-Aldrich), 6 mmol glucose, and with or without 0.1 μg/ml exosomes for 15, 30 and 60 minutes. Cellular ATP concentration was measured using ATPlite 1step luminescence ATP detection assay system (PerkinElmer, Zaventem, Belgium).

Unit Activity Definition

1 unit enzyme activity of GAPDH (AF261085.1) is defined as the activity required for the production of 1 μmole of product per minute.

Exosomes are found to contain 1.1 μU GAPDH activity / μg protein, 3.59 μU PGK (L00159.1) activity / μg protein and 5.5 μU PKm2 (M23725.1) activity / μg protein.

ANTI-OXIDATIVE ACTIVITY

Anti-oxidative activity may be detected in therapeutic exosomes by detection of any one or more of the proteins or their activities, as set out in Table D4, thereby identifying them as therapeutic exosomes.

Table D4

Protein	**GenBank Accession Number/ NCBI Reference Sequence (RefSeq)**	**Synonyms**
GAPDH	AF261085.1, AAF99678.1	
PGD (6-phosphogluconate dehydrogenase)	BC000368.2, AAH00368.1	
PXDN	AF200348.1, AAF06354.1	
PRDX1	BC021683.1, AAH21683.1	
PRDX6	D14662.1, BAA03496.1	
Catalase	AY028632.1, AAK29181.1	

Table D4. Anti-Oxidative Activity

EXTRACELLULAR MATRIX (ECM) MODIFYING ACTIVITY

Extracellular matrix (ECM) modifying activity may be detected in therapeutic exosomes by detection of any one or more of the proteins or their activities, as set out in Table D5, thereby identifying them as therapeutic exosomes.

Table D5

Protein	**GenBank Accession Number/ NCBI Reference Sequence (RefSeq)**	**Synonyms**
MMP1	X54925.1, CAA38691.1	
MMP2	NM_001127891.1, NP_001121363.1	
MMP3	X05232.1, X05232.1	
MMP10	X07820.1, CAA30679.1	
TIMP1	NM_003254.2, NP_003245.1	
TIMP2	NM_003255.4, NP_003246.1	
TIMP3	NM_000362.4, NP_000353.1	

Table D5. Extracellular Matrix (ECM) Modifying Activity

NT5E (CD73) ECTO-5'-ECTONUCLEOTIDASE ACTIVITY

NT5E (CD73) ecto-5'-ectonucleotidase activity may be detected in therapeutic exosomes by detection of any one or more of the proteins or their activities, as set out in Table D6, thereby identifying them as therapeutic exosomes.

Furthermore, as exposing cells to exosomes having NT5E (CD73) ecto-5'-ectonucleotidase activity in the presence of AMP will induce phosphorylation of AKT and ERK1/2, detection of phosphorylated AKT (i.e., phosphorylated AKT1, phosphorylated AKT2 or phosphorylated AKT3) or phosphorylated ERK1 or phosphorylated ERK2 (or any combination of these) in or of the exosome, may be used as a means of detecting NT5E (CD73) ecto-5'-ectonucleotidase enzyme activity in the exosome, thereby identifying it as a therapeutic exosome.

Table D6

Protein	**GenBank Accession Number/ NCBI Reference Sequence (RefSeq)**	**Synonyms**
Ecto-5'-ectonucleotidas	X55740.1, CAA39271.1	NT5E, CD73
Ecto-apyrase	S73813.1, AAB32152.1	CD39
AKT1, phosphorylated form	NM_001014432, NP_001014431	
AKT2, phosphorylated form	NM_001626, NP_001617	
AKT3, phosphorylated form	NM_005465, NP_005456	
ERK1, phosphorylated form	NM_001040056, NP_001035145	MAPK3
ERK2, phosphorylated form	NM_002745, NP_002736	MAPK1

Table D6. NT5E (CD73) ecto-5'-ectonucleotidase Activity

CD73 Assay

CD73 (NT5E) enzymatic activity in exosome was determined by incubating 2.5 μg of exosome in 100 μl Tris buffer pH 7.4 containing 50 μM AMP (Sigma-Aldrich, St Louis, MO).

The amount of phosphate ions released from the hydrolysis of AMP was then determined by Colorlock Gold kit (Innova Biosciences, Cambridge, UK) as per manufacturer's instruction.

Unit Activity Definition

1 unit Ecto-5'- ectonucleotidase enzyme activity is defined as the activity required for the production of 1 μmole of product per minute.

Exosomes are found to contain 22.04 μU Ecto-5'- ectonucleotidase activity / μg protein.

ION HOMEOSTASIS ACTIVITY

Ion homeostasis activity may be detected in therapeutic exosomes by detection of any one or more of the proteins or their activities, as set out in Table D7, thereby identifying them as therapeutic exosomes.

Table D7

Protein	**GenBank Accession Number/ NCBI Reference Sequence (RefSeq)**	**Synonyms**
PMCA1	P20020	
PMCA4	P23634	
Ca2+-dependent ATPase activity ATP2B1	J04027.1, AAA74511.1	
Ca2+-dependent ATPase activity ATP2B4	M25874.1, AAA50819.1	

Table D7. Ion Homeostasis Activity

CHAPERONE ACTIVITY

Chaperone activity may be detected in therapeutic exosomes by detection of any one or more of the proteins or their activities, as set out in Table D8, thereby identifying them as therapeutic exosomes.

Table D8

Protein	**GenBank Accession Number/ NCBI Reference Sequence (RefSeq)**	**Synonyms**
HSP60	M34664.1, AAA36022.1	
HSP70	AB023420.1, BAA75062.1	
HSP90	M27024.1, AAA63194.1	

Table D8. Chaperone Activity

IMMUNOMODULATORY ACTIVITY

A therapeutic exosome may display immunomodulatory activity.

Regulation of Adaptive Immunity by Innate Immune Cells (reviewed 1-3)

The immune system exists primarily to protect the organism against disease by destroying non-self cells or tissues, neutralizing infectious or toxic materials and removing cell debris e.g. dead or dying cells. In vertebrates, the immune system consists of the innate and adaptive immune systems. Innate immune cells include dendritic cells, macrophages and neutrophils and these cells are genetically programmed to recognize invariant features of infectious/toxic materials, stress signals, dead/dying cells, or non-self cells. It is evolutionarily conserved and provides the first line of defense. Adaptive immune cells are the T and B lymphocytes. They adapt to each foreign, toxic or non-self agent by generating receptors specific against these materials de novo to target them for neutralization or destruction. On the other hand, innate immune cells recognize conserved molecular signatures within microbial pathogens referred to commonly as pathogen-associated molecular patterns and stress signals through germline-encoded pattern recognition receptors (PPRs) such as include Toll-like receptors (TLRs), retinoic acid-inducible gene 1-like RNA helicases, nucleotide binding oligomerisation domain-like receptors, and C-type lectin receptors. The activation of adaptive immune cells to generate specific receptors against infectious or toxic agents is complex and modulated by the innate immune cells. Among the PPRs, the TLRs are the best characterized PPRs with regards to modulation of adaptive immune responses such as production of IgM, IgG and IgA antibody, and activation of T_H1, T_H17 $CD4^+$, and $CD8^+$ T cells.

TLRs are highly conserved type 1 integral membrane glycoproteins. In human, ten TLRs have been identified to date. All TLRs, except TLR3, signal through the MyD88-dependent pathway either directly or in combination with TIRAP, ultimately leading to activation and nuclear translocation of NFκB, activating protein 1 (AP1), or both of these, leading to the production of inflammatory cytokines. TLR3 and TLR4 initiate the MyD88-independent, TRIF-dependent signalling pathway leading to the activation and nuclear translocation of either NFκB or IRF3 to produce inflammatory cytokines and type 1 interferon, respectively. Different TLRs recognise different classes of pathogen-associated

molecular patterns, e.g. TLR4 recognises bacterial cell-surface lipopolysaccharides, TLR1 and TLR2 recognise peptidoglycans, TLR2 and TLR6 recognise lipoproteins, TLR3 recognises viral double-stranded RNA, TLR7 and TLR8 recognise viral single-stranded RNA, TLR9 recognises bacterial and viral CpG, TLR5 recognises flagellin.

Endogenous Ligands of TLRs (reviewed 4)

Despite the overwhemingly importance of TLRs in mediating host immunity against infectious agents and their products, activation of TLR signaling by endogenous ligands to trigger a sterile inflammatory response is increasingly viewed as an important function of TLRs. To date, numerous endogenous ligands for the different TLRs have been identified (Table D9 below). TLRs have been implicated in ischemia and reperfusion injury, tissue repair and regeneration, autoimmune diseases, and tumorigenesis and tumour progression.

TLR2	Biglycan, endoplasmin, HMGB1, HSP60, HSP70, human cardiac myosin, hyaluronan and monosodium urate crystals
TLR3	mRNA
TLR4	Biglycan, CD138, α-crystallin A chain, β-defensin 2, endoplasmin, fibrinogen, fibronectin, heparan sulphate, HMGB1, HSP22, HSP60, HSP70, HSP72, hyaluronan, monosodium urate crystals, OxPAPC, resistin, S100 proteins, surfactant protein A and tenascin-C
TLR7	RNA and small interfering RNA (siRNA)
TLR8	Human cardiac myosin and small interfering RNA (siRNA)
TLR9	DNA and HMGB1

Table D9. TLRs and their endogenous ligands. Highlighted ligands are also found to be present in MSC exosomes.

Immune Modulatory Activities of MSCs

MSCs display immunomodulatory properties *in vitro* such as inhibition of T-cell proliferation induced by alloantigens, mitogens and CD3-ligation, inhibit the proliferation of B cells, and the activity of natural killer cells[5]. Clinical trials testing the therapeutic efficacy of MSC-based intervention for human GVHD[6] and Crohn's disease[7] have been encouraging.

However the underlying mechanism for the immunomodulatory properties of MSCs remains poorly understood. Increasingly, it is thought that these properties are mediated by paracrine secretion from MSCs[8].

In 2008, our group demonstrated that culture medium conditioned by human embryonic stem cell-derived MSCs (hESC-MSCs) reduced relative infarct size in a pig and mouse model of ischemia/reperfusion injury[9] and improved cardiac function in pig model of chronic ischemia[10], and the active component has a presumptive diameter of 50-200 nm. This component was subsequently purified by HPLC and identified as an exosome[11]. Proteomic analysis revealed that these exosomes contained endogenous ligands for TLR2 and TLR4 (table 1). Therefore, MSC exosomes have the potential to activate TLR2 and TLR4 and contribute to the immunomodulatory activities of MSCs.

Immunomodulatory activity may be detected in therapeutic exosomes by detection of any one or more of the proteins or their activities, as set out in Table D10, thereby identifying them as therapeutic exosomes.

	Variant	GenBank Accession Number	NCBI Reference Sequence (RefSeq)
Biglycan		AK092954	NM_001711
Endoplasmin		AY040226	NM_003299
HSP60		M34664.1, AAA36022.1	
HSP70		AB023420.1, BAA75062.1	
β-defensin 2		AJ000152	NM_004942
Fibrinogen	alpha		NM_021871
	beta		NM_001184741
	gamma		NM_000509
Fibronectin			NM_212482
Tenascin-C			NM_002160
S100 proteins	S100A11	D38583	NM_005620
	S100A13	AK097132	NM_001024212
	S100A8	BC005928	NM_002964
	S100A9	BC047681	NM_002965
	S100P	X65614	NM_005980

Table D10

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.

The exosome may be produced or isolated in a number of ways. Such a method may comprise isolating the exosome from a mesenchymal stem cell (MSC). Such a method may comprise isolating the exosome from an mesenchymal stem cell conditioned medium (MSC-CM). The exosome may be isolated for example by being separated from non-associated components based on any property of the exosome. For example, the exosome may be isolated based on molecular weight, size, shape, composition or biological activity. 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. One or more biological activities of the exosome may be used to track its activity during fractionation of the mesenchymal stem cell conditioned medium (MSC-CM).

IMMUNOMODULATORY ACTIVITY ASSAY

Immunomodulatory activity of exosomes may be assessed by using THP-1 cells and genetically modified THP- cell line/HEK reporter cell lines. Such cell lines are described in the Examples below.

Immunomodulatory activity of exosomes may be assessed using unmodified THP-1, by assaying the ability of the exosome to induce cytokine production. This is described in detail at Example 23 below. Alternatively cytokine production may be assessed using surrogate reporter cell lines as described in the Examples. For example, a THP1-XBlue with a stably transfected secreted embryonic alkaline phosphatase (SEAP) reporter gene under the transcriptional control of NF-kB promoter or a THP1-XBlue-defMYD that is deficient in MyD88 activity may be used, as described in Example 19.

Immunomodulatory activity of exosomes may also be assayed by their ability to inhibit proliferation of Concanavalin A-activated lymphocytes. This is described in detail in Example 28.

Other methods of assaying immunomodulatory activity of exosomes may be used. These include exposing CD4+ T cells to exosome-treated monocytes e.g. THP-1 to determine if they enhance Tregs or Th17 production. This is described in detail in Example 26.

EXOSOME 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 known in the art and are described for example in Example 1 of WO 2009/105044.

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 exosome, in particular a therapeutic exosome, 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 of WO 2009/105044.

Oxidative Stress

The exosome, in particular a therapeutic exosome, 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 exosome, 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 exosome, 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 exosome, in particular a therapeutic exosome, 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 of WO 2009/105044.

Exosome Size

The exosome 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 exosome size may then be determined by tracking segregation of component proteins with SDS-PAGE or by a biological assay.

Assay of Exosome Size by HPLC Dynamic Light Scattering

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 were 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) were connected in series following the UV-visible detector. The last three detectors were from Wyatt Technology Corporation (California, USA) and were operated by the ASTRA software. The components of the sample were separated by size exclusion i.e. the larger molecules will elute before the smaller molecules. The eluent buffer used was 20 mM phosphate buffer with 150 mM of NaCl at pH 7.2. This buffer was filtered through a pore size of 0.1 μm and degassed for 15 minutes before use. The chromatography system was 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 was set at 220 ηm and the column was oven equilibrated to 25oC. The elution mode was isocratic and the run time was 40 minutes. The volume of sample injected ranged from 50 to 100 ml. The % area of the exosome peak vs. all other peaks was integrated from the UV-visible detector. The hydrodynamic radius, Rh was computed by the QELS and Dawn 8 detectors. The highest count rate (Hz) at the peak apex was taken as the Rh. Peaks of the separated components visualized at 220 ηm were collected as fractions for further characterization studies.

Assay of Molecular Weight by SDS-PAGE

The exosome may have a molecular weight of greater than 100 kDa. For example, the exosome may be such that most proteins of the exosome 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 exosome with less than 500 kDa molecular weight may segregate into the greater than 500 kDa molecular weight retentate fraction. This indicates that the exosome may have a molecular weight of more than 500 kDa.

Assay of Molecular Weight by Biological Activity

The exosome may have a molecular weight of more than 1000 kDa. For example, the exosome 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 exosome 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 of WO 2009/105044.

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 exosome (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 exosome has a molecular weight which is higher than the relevant cutoff of the membrane (e.g., greater than 1000 kDa).

Exosome Molecular Weight

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

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

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 exosome 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 of WO 2009/105044.

Composition

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

For example, the exosome 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 exosome 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 and gene products which may be comprised in an exosome are described in for example WO 2009/105044.

EXOSOME

The active component in the secretion such as an exosome that confers cardioprotection against the reperfusion injury may be isolated by means known in the art and described for example in WO 2009/105044. 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 and subsequently found to be secreted by many cells types including various haematopoietic cells, tumours of haematopoietic or non-haematopoietic origin and epithelial cells. They are distinct entities from the more recently described 'ribonuclease complex' also named exosome.

Exosomes may be defined by morphological and biochemical parameters. Accordingly, the exosomes 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. 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. Exosomes have been used in immunotherapy for treatment of cancer.

USES OF THERAPEUTIC EXOSOMES FROM MESENCHYMAL STEM CELLS

The exosomes including therapeutic exosomes may be used as a substitute for an MSC or MSC-CM, as described above and below.

In particular, the therapeutic exosomes 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 therapeutic exosomes from mesenchymal stem cells. Thus, any uses of mesenchymal stem cells will equally attach to therapeutic exosomes 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, therapeutic exosomes from MSCs may be used to treat such diseases.

Therapeutic exosomes 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 therapeutic exosomes 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 therapeutic exosomes 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 EXOSOMES 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 therapeutic exosomes 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 therapeutic exosomes 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 therapeutic exosomes 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 therapeutic exosomes 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 therapeutic exosomes 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 therapeutic exosomes 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 therapeutic exosomes 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 comprised in an therapeutic exosome, the method comprising obtaining a therapeutic exosome as described, and isolating the polypeptide from the therapeutic exosome.

DELIVERY OF THERAPEUTIC EXOSOMES

The therapeutic exosomes 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 therapeutic exosome 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 therapeutic exosomes to a target.

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

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

We further provide for the use of such a delivery system in a method of delivering a therapeutic exosome 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 therapeutic exosomes at the appropriate interval, in order to achieve optimal treatment.

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

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

The therapeutic exosomes may be used for the treatment or prevention of dermatological conditions e.g. psoriasis. Long term delivery of therapeutic exosomes 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 therapeutic exosomes 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 therapeutic exosomes 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 therapeutic exosomes 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 therapeutic exosomes 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 therapeutic exosomes 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 therapeutic exosomes 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 exosomes, including therapeutic exosomes, 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 exosomes 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 exosomes 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 exosomes 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

Example 1. Materials and Methods - Preparation of Exosomes

Exosomes were purified from huES9.E1 derived MSCs conditioned media (CM) using HPLC as described earlier. In brief, CM collected from MSCs culture was concentrated 50x by tangential flow filtration (TFF) using a membrane with a 100 kDa MWCO (Sartorius, Goettingen, Germany).

After that, CM was passed through chromatography columns (TSK Guard column SWXL, 6x40 mm and TSK gel G4000 SWXL, 7.8x300 mm, Tosoh Corp., Tokyo, Japan).

Exosomes were collected from the first peak of the elution, concentrated using 100 kDa MWCO filter (Sartorius). Exosomes were filtered with a 0.22 μm filter before storage or use.

Example 2. Materials and Methods - LC MS/MS

Proteins in two ml of dialyzed exosomes were reduced, alkylated and tryptic digested as described (20).

The samples were 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 were then dried to about 10% of their initial volumes by removing organic solvent in a vacuum centrifuge.

To reduce sample complexity, offline peptide fractionation was carried out with a HPLC system (Shimadzu, Japan) through a Polysulfoethyl SCX column (200 mm x 4.6 mm) (PolyLC, USA).

Mobile phase A (5 mM KH4PO4 + 30% acetonitrile) and mobile phase B (5 mM KH4PO4 + 30% acetonitrile + 350 mM KCl) at 1 ml/min.

Eight fractions were collected and dried with a vacuum centrifuge.

Fractionated samples were loaded into the auto sampler of a Shimadzu micro HPLC system coupled online to a LTQ-FT Ultra linear ion trap mass spectrometer (Thermo Electron, Bremem, Germany) fitted with a nanospray source.

Injected peptides were trapped and desalted in a Zorvax 300SB-C18 enrichment column (5 mm x03 mm, Agilent Technologies, Germany) and eluted into a nano-bored C18 packed column (75 μm x 100 Å, Michrom Bioresources, Auburn, CA).

A 90 minute gradient at a constant flow rate of 20 μl/min with a splitter to an effective flow rate of 200 ηl/min was used to elute the peptides into the mass spectrometer.

The LTQ was 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 were combined into a single mascot generic file by a home-written program.

Protein identification was achieved by searching the combined data against the IPI human protein database (version 3.34; 69,164 sequences, 29,064,825 residues) via an in-house Mascot server (Version 2.2.04, Matrix Science, UK).

The search parameters were: a maximum of 2 missed cleavages using trypsin; fixed modification was carbaminomethylation of cysteine and variable modification was oxidation of methionine.

The mass tolerances were set to 10 ppm and 0.8 Da for peptide precursor and fragment ions respectively.

Protein identification was accepted as true positive if two different peptides were found to have scores greater than the homology scores.

Example 3. Materials and Methods - Antibody Array

500 µl of non-conditioned media and exosomes from 3 independent preparations were assayed for the presence of cytokines and other proteins using RayBio® Biotin Label-based Human Antibody Array I according to manufacturer's instructions (RayBio, Norcross, GA).

The cytokines and other proteins were considered to be present in the exosomes if the signal intensity is 2 fold higher ($p<0.05$) than that in non-conditioned medium.

Example 4. Materials and Methods - Western Blot Hybridization

12 µg exosomes were separated on 4-12% SDS-polyacrylamide gels and electroblotted onto a nitrocellulose membrane.

The membrane was transferred to the membrane holder of SNAP i.d. system (Millipore, Billerica, MA), blocked and incubated with primary anti-human antibodies, which included mouse anti-GAPDH (1:100 dilution), mouse anti-PGK (1:60), mouse anti-PGD (1:60), rabbit anti-PFKFB3 (1:60), mouse anti-pyruvate kinase (PK, 1:200), mouse anti- 20S proteasome α1-7 (1:200), mouse anti-CD73 (1:60) and mouse anti-CD59 (1:200) .

The blot was then incubated with a horseradish peroxidase-coupled secondary antibody.

The secondary antibody used was goat anti-mouse IgG (1:1250) or donkey anti-rabbit IgG (1:1250).

All antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA except mouse anti-PK which was from Abcam Inc., Cambridge, MA.

The blot was then incubated with HRP-enhanced Chemiluminescent substrate (Thermo Fisher Scientific Inc., Waltham, MA) and then exposed to X-ray film.

Example 5. Materials and Methods - Enzyme Assays - Pyruvate Kinase Assay

5 μg of exosome (in 12 μl) was lysed using a cell extraction kit (Biovision, Mountain view, CA).

The lysed exosome extract was incubated with 50 μl of reaction mix from a commercially available PK assay kit (Biovision).

In this assay, pyruvate produced by PK was oxidized by pyruvate oxidase to produce fluorescences (Ex/Em= 535/587 nm).

The increase in fluorescence intensity is therefore proportional to the amount of pyruvate produced.

Example 6. Materials and Methods - Enzyme Assays - GAPDH and PGK Assay

GAPDH and PGK activity were measured based on their downstream product, ATP in the glycolysis reaction using 2 commercially available kits, KDalert GAPDH assay kit (Ambion Inc., Austin, TX) and ApoSENSOR ADP/ATP ratio assay kit (Biovision).

Briefly, exosomes were lysed using a cell extraction kit (Biovision).

To measure GAPDH activity, 10 μg of lysed exosomes was added to KDalert reaction buffer containing D-glyceraldehyde-3-phosphate, NAD^+ and P_i to form 1, 3-bisphosphoglycerate + NADH+ H^+.

250 U/ml of PGK and 60 μM ADP were then added to convert 1, 3-bisphosphoglycerate and ADP to 3-phosphoglycerate and ATP.

The amount of ATP produced which was proportional to GAPDH activity, was then measured using ATP luciferase assay.

To measure PGK activity, 10 μg of lysed exosomes was added to the 1, 3-bisphosphoglycerate produced from the above assay.

ADP was added to allow the formation of ATP.

ATP amount which is proportional to PGK activity was then quantified using ATP luciferase assay.

Example 7. Materials and Methods - Enzyme Assays - 20S Proteasome Assay

The proteasome activity was measured using a 20S proteasome activity assay kit (Millipore) based on detection of the fluorophore 7-Amino-4 methylcoumarin (AMC) after cleavage from the labeled substrate LLVY-AMC by 20S proteasome in the presence or absence of lactacystin, a specific 20S proteasome inhibitor.

Briefly, 4 µg of exosome was incubated with a reaction buffer containing LLVY-AMC in the presence or absence of 25µM actacystin.

The samples and AMC standards were incubated at 37 °C and fluorescense intensity at Ex/Em= 380/460 nm was monitored for 2 hours.

Example 8. Materials and Methods - Enzyme Assays - CD73 Assay

CD73 (NT5E) enzymatic activity in exosome was determined by incubating 2.5 µg of exosome in 100 µl Tris buffer pH 7.4 containing 50 µM AMP (Sigma-Aldrich, St Louis, MO).

The amount of phosphate ions released from the hydrolysis of AMP was then determined by Colorlock Gold kit (Innova Biosciences, Cambridge, UK) as per manufacturer's instruction.

Example 9. Materials and Methods - Cell assays - Glycolysis

H9C2 cardiomyocytes were plated onto a 96 well plate (poly-lysine coated) at 30,000 cells per well.

After 5 hours, the cells were washed twice with Tyrode's buffer before incubating in Tyrode's buffer containing 20 µmol oligomycin (Sigma-Aldrich), 6 mmol glucose, and with or without 0.1 µg/ml exosomes for 15, 30 and 60 minutes.

Cellular ATP concentration was measured using ATPlite 1step luminescence ATP detection assay system (PerkinElmer, Zaventem, Belgium).

Example 10. Materials and Methods - Cell assays - Adenosine-Mediated Signaling

H9C2 cardiomyocytes were plated onto a 6 well plate at 200,000 cells per well and serum starved overnight.

The cells were then incubated with fresh serum-free medium with or without 1mM theophylline for another hour.

In the meantime, two series of serum-free media were prepared.

One series contained no supplement, 50 μM AMP alone, 0.1μg/ml exosomes alone or a combination of 50 μM AMP and 0.1μg/ml exosomes.

The second series was similar to the first except that the media contained 1mM theophylline.

Both series were incubated at 37°C for 30 minutes.

The first series was used to replace medium of the cells that were being cultured in serum-free medium alone and the second series to those cells that were being cultured in theophylline-containing medium.

After five minutes, the cells were harvested and lysed using a commercially available mammalian cell extraction kit (BioVision) in the presence of a protease and phosphatase inhibitor cocktail 1 (Sigma Aldrich).

Protein concentration was determined by standard Bradford assay.

10 μg of the total proteins were analysed by western blot hybridization using 1:2000 dilution of rabbit anti-pERK 1/2 (Cell Signaling, 9101S), 1:2000 dilution of rabbit anti-AKT (Cell Signaling, 9271S), 1:500 dilution of rabbit anti-ERK1 (Santa Cruz,sc-94) and 1:500 dilution of rabbit anti-AKT (Cell Signaling, 9272S).

Example 11. Materials and Methods - Cell assays - Complement-Mediated Cell Lysis

Briefly, sheep red blood cells (SRBCs) were purchased (Innovative Research, Southfield, MI) and washed three times with phosphate buffered saline (PBS) before resuspending at 1×10^8 cell/ml PBS.

Purified complement components, C5b6, C8 and C9 were purchased from Calbiochem (San Diego, CA), and C7 from Sigma-Aldrich (St Louis, MO).

Assembly of intact C5b-9 on SRBCs was initiated by a 15 min incubation (37 °C) with 1ml each of C5b6 (0.1μg/ml) and C7 (0.4μg/ml) in the presence or absence of exosomes at a final concentration of 0.1 μg/ml.

The SRBCs were then washed and incubated with 1ml each of C8 (0.4μg/ml,) plus C9 (0.4μg/ml) with or without a blocking CD59 antibody at a final concentration of 0.05 μg/ml for an additional 30 min.

After that the SRBCs were centrifuged and the amount of hemoglobin released by the lysed SRBCs in the supernatant was measured by absorbance at 415 nm.

Total (100%) hemolysis was obtained by treating the cells with 1% (w/v) Triton X-100.

Example 12. Results - Proteomic Profiling of Exosome

Exosome was purified from culture medium conditioned by HuES9.E1, a human ESC-derived mesenchymal stem cells[13] by HPLC as previously described[9,10]. Proteomic profiling using mass spectrometry and antibody approaches was performed as previously described[9-11] on 3 independently prepared batches of HPLC-purified exosomes. A total of 866 proteins were detected and these proteins were denoted by their gene symbol to facilitate analysis (Table E1). Of these, 318 gene products were found in the 739 proteins previously identified in the unfractionated conditioned medium[9,10] (Figure 1).

Based on data analysis of 15 proteomic analyses carried out on exosomes purified from cultured cells and from biological fluids, Thery et al. had observed that a set of about 17 proteins, namely glyceraldehyde 3-phosphate dehydrogenase (GAPDH), pyruvate kinase (PK), eukaryotic translation elongation factor 1A1 (EEF1A1), milk fat globule EGF factor 8 protein (MFGE8), tetraspanins, 14-3-3 proteins, Gα proteins, clathrin, Alix (PDCD6IP), MHC class1, annexins (ANX), Rab proteins, ezrin(VIL2), radixin(RDX) and moesin (MSN)(ERM), actin, tubulin, HSP70 and HSP90 were found to be present in at least 50% of the exosomes that were characterized[2]. Not unexpectedly, most of these proteins were also found in the proteome of the HPLC-purified MSC exosomes (Table E1). Also consistent with the

endosomal origin of exosomes, we detected the presence of endosome-associated proteins such as Alix (PDCD6IP) and Rab (Table E1).

A2M	BSG	COMP	FBLN1	HMGCS2	KRT15	MMP2	PRR4	RPS4X	TGFB1
ABI3BP	C11orf59	COPB1	FBN1	HNRNPA1	KRT16	MMP3	PRSS23	RPS5	TGFB2
ACAA2	C1orf78	COPS3	FBN2	HP	KRT17	MOS	PSMA1	RPSA	TGFBI
ACAT2	C1R	COPS4	FBXW8	HPX	KRT18	MPO	PSMA2	RRAS2	TGM2
ACLY	C1S	COPS8	FEN1	HRSP12	KRT19	MPZL1	PSMA3	RTN4	TGOLN2
ACSL1	C20orf114	CPS1	FER1L3	**HSP90AA1**	KRT2	MRC2	PSMA4	RUVBL1	THBS1
ACTA1	C3	CREG1	FGA	**HSP90AB1**	KRT27	**MSN**	PSMA5	S100A11	THBS2
ACTA2	C5orf24	CRIPT	FGB	**HSP90B1**	KRT28	MSTN	PSMA6	S100A13	THY1
ACTB	C9orf19	CRTAP	FGF16	**HSPA1A**	KRT3	MXRA5	PSMA7	S100A8	TIMP1
ACTG2	C9orf91	CSF1	FGF18	**HSPA1L**	KRT4	MYADM	PSMB1	S100A9	TIMP2
ACTN1	CACNA2D1	CSF2	FGF19	**HSPA5**	KRT5	MYCBP AP	PSMB10	S100P	TIMP3
ACTN2	CACNA2D4	CSF3	FGFRL1	**HSPA6**	KRT6A	MYH14	PSMB2	SAA4	TKT
ACTN3	CALR	CSPG4	FGG	**HSPA8**	KRT6B	MYH9	PSMB3	SASS6	TLN1
ACTN4	CAND1	CST4	FLG2	HSPB1	KRT6C	MYL6B	PSMB4	SCAMP3	TMBIM1
ACTR1 A	CAP1	CTA-221G9.4	FLJ1319 7	HSPD1	KRT7	MYO1C	PSMB5	SCGB2A 1	TMED10
ACTR2	CAPNS1	CTBP2	FLJ2218 4	HSPG2	KRT72	NBL1	PSMB6	SCYE1	TMED9
ACTR3	CAPZA1	CTNNA1	FLJ3278 4	HTRA1	KRT73	NEFH	PSMB7	SDC1	TMEM16B
ADAM1 0	CASP14	CTNNA2	FLNA	HYI	KRT74	NEK10	PSMB8	SDC2	TMEM2
ADAM9	CAT	CTNNB1	FLNB	ICAM1	KRT76	NID1	PSMB9	SDC4	TMEM47
ADAM TS12	CAV1	CTNND1	FLNC	ICAM5	KRT77	NLRP8	PSMC5	SDCBP	TMEM51
AEBP1	CCDC129	CTSG	FLOT1	IDH3B	KRT78	NME1	PSMD11	SEC14L4	TNC
AFM	CCDC64B	CXCL16	FLOT2	IFITM2	KRT79	NOMO1	PSMD14	SEMA5 A	TNFRSF11 B
AGRN	CCL20	CXCL2	FLT1	IFNG	KRT8	NRAS	PSMD6	SEPT2	TNFRSF12 A
AHCY	CCL28	CXorf39	FN1	IFRD1	KRT80	NRG2	PSMD7	SEPT7	TNFRSF1 A
AHNAK 2	CCL7	CYBRD1	FREM3	IFT140	KRT84	NRP1	PTGFRN	SERINC 5	TNFSF18
AHSG	CCR4	DBF4B	FST	IGF2R	KRT9	NT5E	PTK7	SERPIN A1	TPBG
AKR1B 1	CCR5	DCD	FTL	IGFBP3	LACRT	NTF4	PTPRK	SERPIN B3	TPI1
AKR7A 2	CCT5	DCHS2	FUCA2	IGFBP4	LAMA4	NUSAP1	PTRF	SERPIN E1	TRAP1
ALB	CCT6A	DCLK2	GALNT5	IGFBP6	LAMB1	OBFC1	PTTG1IP	SERPIN E2	TREM1
ALCAM	CD109	DCN	GANAB	IGFBP7	LAMC1	ODZ3	PTX3	SERPIN F1	TREML2P
ALDH2	CD151	DCTN1	**GAPDH**	IGHA1	LAMP1	OFD1	PXDN	SFN	TRIM40

ALDH3A2	CD248	DECR1	GAPDHS	IGHA2	LAMP2	OPRM1	PZP	SFRP1	TRIM41
ALDH6A1	CD276	DEFA1	GARS	IGHG1	LAP3	OSM	QPCTL	SFRP4	TSN
ALDH7A1	CD40LG	DIP2B	GAS6	IGHG2	LCN1	OTC	QSOX1	SHANK3	TSNAX
ALDH9A1	CD44	DIRAS2	GDF1	IGHG4	LCN2	OXNAD1	RAB10	SLAIN1	TSPAN14
ALDOA	CD47	DKFZp686D0972	GDF11	IGHM	LDHA	OXTR	RAB11B	SLC16A1	TSPAN4
ALDOB	CD59	DKK1	GDF15	IGJ	LDHAL6B	P4HB	RAB14	SLC16A3	TSPAN6
ALDOC	CD63	DKK3	GDF3	IGKC	LDHB	PAICS	RAB15	SLC1A4	TSPAN9
ALOX12P2	CD81	DMBT1	GDF5	IGKV1-5	LEPRE1	PAN3	RAB1A	SLC1A5	TSTA3
AMH	CD82	DNASE1L1	GDF9	IGL@	LGALS1	PAPPA	RAB1B	SLC22A2	TTLL3
ANG	CD9	DNPEP	GDI1	IGLV4-3	LGALS3	PARP10	RAB2A	SLC25A10	TTN
ANGPTL2	CDC2L5	DPYS	GDI2	IGSF8	LGALS3BP	PARP16	RAB33B	SLC25A13	TTYH3
ANGPTL4	CDC42	DPYSL2	GFRA3	IL10	LGALS8	PARVG	RAB35	SLC2A1	TUBA1A
ANPEP	CDH13	DSP	GLDC	IL11	LGR6	PC	RAB39B	SLC2A3	TUBA1B
ANXA1	CDIPT	DULLARD	GLUD1	IL13	LIF	PCOLCE	RAB5A	SLC38A2	TUBA1C
ANXA11	CDK5R2	ECM1	GNA13	IL15RA	LMNA	PDCD6	RAB5B	SLC38A3	TUBB
ANXA2	CEACAM8	EDA	GNAI2	IL17B	LOC124220	PDCD6IP	RAB5C	SLC39A14	TUBB2A
ANXA2P1	CFB	EDG2	GNAL	IL17RA	LOC283523	PDGFA	RAB6A	SLC3A2	TUBB2C
ANXA3	CFI	EDIL3	GNAS	IL19	LOC284297	PDGFC	RAB7A	SLC44A1	TUBB3
ANXA4	CFL1	EEA1	GNAT3	IL1F9	LOC388344	PDGFRB	RAB8A	SLC44A2	TUBB6
ANXA5	CFL2	EEF1A1	GNB1	IL1RAP	LOC389827	PDIA3	RAB8B	SLC7A10	UBA52
ANXA6	CFTR	EEF1G	GNB2	IL1RAPL1	LOC442497	PEBP1	RAC1	SLC7A5	UBB
ANXA7	CHMP2A	EEF2	GNB4	IL1RL2	LOC653269	PFAS	RAC2	SMAD4	UBE1
AP1S1	CHRDL1	EFEMP2	GNG12	IL22RA1	LOC727942	PFKFB3	RAD21	SMARCA4	UBE2N
APEH	CHST12	EHD1	GNPDA1	IL23A	LOC728320	PFN1	RALA	SMC1A	UGP2
APOA1	CITED1	EHD2	GOT2	IL3	LOC728378	PFN2	RAN	SORT1	UNC13B
APOE	CLASP2	EHD4	GPC1	IL5	LOC730013	PGAM2	RAP1A	SOS1	UNC45A
APP	CLDN1	EIF4A1	GPC5	IL6	LOXL2	PGD	RAP1B	SPACA1	VAMP3
ARF1	CLEC11A	EMILIN1	GPI	IL6ST	LRP1	PGK1	RAP1B	SPARC	VANGL1
ARF4	CLIC1	ENC1	GPR112	IL7	LRP6	PGLYRP	RAP2C	SPOCK1	VASN

						2			
ARF5	CLIC6	ENG	GREM1	IL8	LRRFIP2	PIGR	RARRES 1	SPRY4	VAT1
ARHGA P18	CLPX	ENO1	GRM2	INHBA	LTBP1	PIP	RASA1	SPTAN1	VCAN
ARHGA P23	CLSTN1	ENO2	GRM3	INHBB	LTBP2	PITX3	RASA4	SPTBN1	VCL
ARHGD IA	**CLTA**	ENO3	GRM7	INSR	LTF	**PKM2**	RB1CC1	SPTBN4	VCP
ARHGE F1	**CLTC**	ENTPD4	GSN	IQGAP1	LYAR	PLAU	RCOR2	SRGN	VEGFC
ARL6IP 5	**CLTCL1**	EPB41L3	GSTM1	ITGA11	LYZ	PLEC1	RDH5	SRI	VIL1
ARMS2	CLU	EPHA2	GSTM2	ITGA2	MAMDC 2	PLEKHG 3	RFTN1	SRPX2	**VIL2**
ARPC3	CMIP	EPO	GSTM5	ITGA3	MAP1A	PLOD1	RGN	ST6GAL NAC6	VIM
ARPC4	CNGB1	EPX	GSTO1	ITGA4	MAP2K6	PLOD2	RHOC	STAT1	VTI1A
ARPC5	CNOT6	ESM1	GSTP1	ITGA5	MAP3K1	PLOD3	RMND5 A	STC1	VTN
ASH1L	CNTN1	ETFB	GTPBP2	ITGAL	MARCK S	PLP2	RNF123	STC2	WDR49
ASL	COL12A1	F2R	GYLTL1 B	ITGAV	MARCK SL1	PLSCR3	RNF40	STOM	WDR52
ATP1A1	COL14A1	F3	GZMA	ITGB1	MAT1A	PLTP	RPL10A	STOML3	WNT5A
ATP1B3	COL18A1	F8	H2AFV	ITGB5	MBD3	PLUNC	RPL12	STX12	YBX1
ATP2B1	COL1A1	FADD	H2AFX	ITIH2	MCC	PNO1	RPL15	STX2	**YWHAB**
ATP2B4	COL1A2	FAH	HBB	ITIH4	MCM10	PODN	RPL18	SURF4	**YWHAE**
ATP5A1	COL2A1	FAM108 A1	HBE1	ITPR2	MDH1	POLN	RPL23	SVEP1	**YWHAG**
ATP5B	COL3A1	FAM129 B	HDAC5	JUP	MDH2	POSTN	RPL29	SYT1	**YWHAQ**
ATP8B3	COL4A1	FAM29A	HERC5	KIAA0146	ME1	POTE2	RPL35A	SYT9	**YWHAZ**
ATRN	COL4A2	FAM3B	HGF	KIAA0256	MECP2	PPIA	RPLP0	TAAR2	ZBTB4
ATXN1	COL4A3	FAM64A	HISPPD2 A	KIAA0467	MET	PPIB	RPS10	TAGLN	ZNF134
AXL	COL5A1	FAM71F 1	HIST1H2 AE	KIAA1881	MFAP4	PPME1	RPS16	TALDO1	ZNF503
BASP1	COL5A2	FAP	HIST1H2 BA	KPNB1	**MFGE8**	PPP1CC	RPS18	TAS2R6 0	ZNF614
BDNF	COL6A1	FASN	HIST1H2 BL	KRT1	MFSD2	PRDM16	RPS2	TCN1	
BGN	COL6A2	FAT	HIST1H4 H	KRT10	MIF	PRDX1	RPS24	TF	
BHMT2	COL6A3	FAT2	HIST2H2 BE	KRT13	MMP1	PRDX6	RPS27A	TFG	
BRMS1	COL7A1	FAT4	**HLAA**	KRT14	MMP10	PRNP	RPS3	TFRC	

Table E1: Proteomic profile of 3 independently prepared exosomes as determined by LC MS/MS and antibody arrays. Black font - Identified by LC MS/MS. Underline - Identified

by antibody arrays. Grey shade - Identified by LC MS/MS and was found to be present in at least 50% of exosomes characterized[2]

Example 13. Results - Computational Analysis of Exosome Proteome

To better understand the biological significance of the proteins in the exosomes, functional clustering of the 866 proteins into biological processes was performed using PANTHER (Protein ANalysis THrough Evolutionary Relationships) analytical software[14,15]. The observed frequency of genes from the exosome proteome in each biological process was compared with the reference frequency of genes in the NCBI database for that biological process. The 866 gene products could be clustered into 32 biological processes that were over-represented ($p<0.001$) and 3 that were under-represented ($p<0.001$) (Figure 2).

These biological processes could be further classified into several activities associated with exosome biology e.g. communication, cellular motility, inflammation and exosome biogenesis. As exosomes are generally postulated to function as vehicles of intercellular communication and morphogen signaling[4,16] and mediators of immune activity[2], the involvement of exosome proteins in signal transduction pathways, cell structure, cell motility and immune responses was not unexpected. A fourth class of processes that we tentatively termed as "exosome biogenesis" essentially reflected the biogenesis and release of exosomes through the involvement of endosomes, ESCRT-mediated sorting and the formation of multivesicular bodies, and fusion with the plasma membrane. Therefore, proteins such as those involved in trans-golgi network, intracellular protein traffic, general vesicle transport, endocytosis, receptor-mediated endocytosis, other protein targeting and localization and exocytosis are not unexpectedly enriched in exosomes. Other biological processes that we classified as tissue repair and regeneration include those that are involved in the development and differentiation of mesodermal and ectodermal tissues including skeletal development and angiogenesis. These biological processes are consistent with the differentiation potential of mesenchymal stem cells[17] and reflected the cellular origin of the exosomes. In our classification of metabolism, we included processes involved in catabolic or anabolic metabolisms that produce energy and building blocks for growth and regeneration. Blood clotting was also a significantly over-represented biological process that we postulated may be important in ameliorating tissue injury. All three under-represented biological processes were not unexpectedly involved in regulating gene expression at the transcriptional level. Most proteins involved in these processes tend to be transported efficiently into the nucleus, making

them less likely to be associated with exosomes. We also observed that 421 proteins found in the conditioned medium (CM) were not present in the exosome (Figure 1) and these proteins could be functionally clustered into eleven over-represented and two under-represented biological processes. Of the over-represented processes, six were also found in the clustering of the 866 proteins found in exosomes while the remaining five, namely amino acid activation, protein folding, chromatic packaging and remodeling, protein complex assembly and mRNA splicing were not. The two under-represented biological processes were also found to be under-represented for the exosome proteins. The diverse array of proteins in MSC exosomes indicated that they have the potential to participate in a wide spectrum of biochemical and cellular activities. To test this hypothesis, we selected those proteins for which assays to test their biochemical and cellular activities are available, and that together would provide an indication for the wide diversity in activities of exosomes.

Example 14. Results - Exosome Enhanced Cellular ATP Production Through Glycolysis

One prominent feature of the exosome proteome was the presence of all five enzymes in the ATP generating stage of the glycolysis (Figure 3A): glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), phosphoglucomutase (PGM), enolase (ENO) and pyruvate kinase m2 isoform (PKm2). Of these, the three enzymes that generate either ATP or NADH namely GAPDH, PGK, and PKm2 were further confirmed to be present by immunoblotting (Figure 3B). Their enzymatic activities were determined to be 1.1 μU, 3.59 μU and 5.5 μU per μg protein respectively (Figure 3C) where 1 unit (U) of enzyme activity is defined as the activity required for the production of 1 μmole of product per minute.

In addition, exosome contains PFKFB3 which converts fructose 6-phosphate to fructose 2, 6-bisphosphate. PFKFB3 is one of four PFKFB isoforms encoded by four different genes, PFKFKB1, 2, 3 and 4. PFKFBs are responsible for maintaining the cellular level of fructose-2,6-bisphosphate, a powerful allosteric activator of phosphofructokinase[18] which catalyses the commitment to glycolysis. They are thought to be responsible for the high glycolytic rate or "Warburg effect" in cancer cells[19]. The kinase activity of PFKFB3 is upregulated by phosphorylation by protein kinases such as cAMP-dependent protein kinase and protein kinase C. Mass spectrometry analysis revealed the presence of PFKFB3 in the exosome and immunoblotting further demonstrated that this enzyme was phosphorylated (Figure 3A).

The presence of ATP-generating glycolytic enzymes and phosphorylated PFKFB3 in exosomes predicted that exposure of cells to exosome could increase glycolytic flux and increase ATP production. To test this, we determined if these exosomes could increase ATP synthesis in oligomycin-treated H9C2 cells. Since oligomycin inhibits mitochondrial ATPase[20], oligomycin-treated cells would have to utilize glycolysis as their major source of cellular ATP. Consistent with the presence of glycolytic enzymes and PFKFB3, and our previous demonstration that H9C2 cells could internalize MSC exosomes[12], exosomes increased ATP level in oligomycin-treated cells by 75.5 ± 28.8% or 55.8 ± 16.5% in 15 to 30 minutes of exposure to exosomes respectively through increased glycolysis.

Example 15. Results - 20S Proteasome in Exosome is Enzymatically Active

Mass spectrometry analysis of MSC exosomes not only detected the presence of all seven α- (PMSA1-7) and all seven β-subunits (PMSB1-7) of the 20S core particle, but also the three beta subunits of "immunoproteasome", PMSB8 (β5i or LMP7), PMSB9 (β1i or LMP2), PMSB10 (β2i or LMP10) gene product [21]. The presence of some of the 20S proteasome peptides was further confirmed by western blot hybridization (Figure 4A). The presence of all seven α- and all seven β-subunits of the 20S core particle suggest that MSC exosomes contain intact 20S proteasome complexes and therefore potentially possess 20S proteasome enzymatic activity.

Consistent with this, MSC exosome was able to degrade short fluorogenic peptides with an enzymatic activity of 5.00 μU/μg protein and this degradation was inhibited by lactacystin, a specific proteasome inhibitor (Figure 4B). We also observed that unlike the glycolytic enzyme assays described above, 20S proteasome activity in the exosomes could be detected without lysing the exosomes. This suggested that the 20S proteasome may be present on the surface and not in the lumen of exosomes

It was previously observed that 20S proteasome preferentially assembles in an "end-on" configuration on phosphatidylinositol lipid monolayer, ER and Golgi lipid films[22] such that entrance to 20S proteasome is perpendicular to membrane. Therefore, this together with our observations suggests that 20S proteasomes in the exosomes were attached to external membrane surface and their entrances were perpendicular to the exosome membrane.

Example 16. Results - Exosome Phosphorylated ERK and AKT via NT5E (CD73) Ecto-5'-Ectonucleotidase

Ecto-5'-nucleotidase (NT5E or CD73) together with ecto-apyrase (CD39) enzymatically convert precursor nucleotides into adenosine. During cellular injury, cells release ATP and ADP[23]. CD39 hydrolyzes extracellular ATP and ADP to AMP which is then degraded to adenosine by ecto-5'-nucleotidase.

Adenosine has a half-life of ten seconds in human blood. It is a powerful vasodilator but is not used clinically as a vasodilator as it is very short acting. It is used for the rapid treatment of supraventricular tachycardias. Adenosine is an endogenous purine nucleoside that modulates many physiological processes through four known adenosine receptor subtypes (A1, A2A, A2B, and A3)[24].

NT5E was found to be present in the MSC exosomes by mass spectrometry analysis and confirmed by immunoblotting (Figure 5A). Their enzyme activities were determined to be 22.04 μU/μg protein. To determine if exosomes could activate adenosine signaling in cells via CD73-mediated hydrolysis of AMP, H9C2 cardiomyocytes were serum starved overnight and then exposed to exosomes and AMP. Cell lysates were then analyzed for phosphorylation of ERK1/2 and AKT (Figure 5B, Figure 5C).

After overnight serum starvation, exposure to exosomes and AMP induced phosphorylation of ERK1/2 and AKT. The phosphorylation of ERK1/2 and AKT was abolished in the presence of theophylline, a non-selective adenosine receptor antagonist, antagonized A1, A2A, A2B, and A3 receptors[25].

Example 17. Results - Exosome Inhibited the Formation of Membrane Attack Complex

The complement system is a part of the innate immune system which complements the function of antibodies. Upon activation, a biochemical cascade is initiated to generate several key products: C3b which binds to the surface of pathogens and enhance phagocytosis of these pathogens; C5a which helps to recruit inflammatory cells by chemotaxis; and C5b which initiates formation of the MAC consisting of C5b, C6, C7, C8, and polymeric C9. MAC deposited on the target cell forms a transmembrane channel which causes subsequent cell lysis. Aberrant activation of the complement pathway is thought to play a deleterious role in ischemia reperfusion injury[26].

MSC exosome was found to contain CD59 by mass spectrometry analysis and this was confirmed by immunoblotting (Figure 6A). Since CD59 inhibits formation of MAC[27], this suggested that exosomes may inhibit complement activation and subsequent complement-mediated cell lysis. Consistent with this hypothesis, exosome inhibited complement-mediated lysis of sheep red blood cells (SRBCs) (Figure 6B). This inhibition was abolished when a CD59 blocking antibody was used to pre-treat the exosomes, showing that CD59 of exosomes is directly involved in the inhibition of complement lysis.

Example 18. Discussion (for Examples 1 to 17)

In this report, we profiled the proteome of 3 independently prepared, HPLC-purified ESC-derived MSC exosome using mass spectrometry and cytokine array and identified 866 proteins. These proteins included many proteins commonly found in other exosomes.

Clustering of these proteins according to their functions suggested that the exosome has the potential to drive many biological processes. To evaluate this hypothetical biological potential, we examined a set of proteins selected on the basis that assays for their biochemical and cellular activities are available, and that as a group, they would illustrate the wide ranging diversity in activities. More importantly, the biochemical and cellular activities of these selected proteins could potentially ameliorate tissue injury in acute myocardial ischemia/reperfusion injury.

The proteome of MSC exosomes contained a diverse array of proteins. A significant fraction are proteins that are involved in the highly regulated and complex intracellular membrane trafficking and sorting through the biosynthetic and endocytotic pathways[28] and the presence of these proteins are probably a reflection of the biogenesis of exosomes which are essentially small bi-lipid membrane vesicles secreted by many cell types. It was previously observed that exosomes from different cellular sources carry a common set of proteins, many of which are reflective of their biogenesis[29]. The complexity in the biogenesis of exosome and selective loading of the protein and RNA cargo load suggest a heavy investment of cellular resources and such commitment must be underpinned by important physiological functions.

Although exosomes have been discovered for more than 30 years, the biological significance of exosome is just starting to be uncovered. It has been implicated in an increasing number of important physiological and pathological processes such as disposal of unwanted protein[1], antigen presentation[30], genetic exchange[31], immune responses[32,33] and

tumor metastasis[32-37]. Together, these observations suggest that as a group or individually, exosomes may modulate many biological processes. The latter possibility is supported by the wide array of proteins and RNAs that has been found in exosomes[12,38]. In addition to the functions of exosomes listed above, we have recently observed that exosomes secreted by MSCs could reduce infarct size in a mouse model of acute myocardial ischemia/reperfusion injury[9]. To investigate and elucidate the underlying molecular mechanism, we undertook a systematic proteomic interrogation of the MSC exosomes to reveal the cellular and biochemical potential of these exosomes and to identify candidate biological activities that could ameliorate tissue injury in acute myocardial ischemia/reperfusion injury[9].

We first determined that the glycolytic enzymes that are responsible for generating ATP and NADH[18], were not only present in the exosomes but were also biochemically active. In addition, the exosomes also contained the active phosphorylated form of PFKB3 that catalyzed the formation of fructose-2,6-bisphosphate, a powerful allosteric activator of phosphofructokinase These observations indicated that MSC exosomes have the potential to restore cellular ATP and NADH through glycolysis, independent of mitochondrial function. Besides being the major site of ATP production, mitochondria is also the major organelle in regulating cell death[39]. Loss of mitochondrial function and subsequent depletion of ATP generally represent the early steps in the cascade leading to cell death during pathological conditions such as acute myocardial ischemia/reperfusion injury. ATP deficiency, a major index of cell viability, is the key consequence of mitochondrial dysfunction. Since MSC exosomes could be internalized by cells[12], exosome has the potential to restore cellular ATP level by increasing cellular content of glycolytic enzymes or phosphorylated PFKB3 in cells with mitochondrial damage. Indeed, when mitochondrial ATPase in H9C2 cells was inhibited by oligomycin, exosomes could increase ATP production in these cells.

The presence of all seven α and β subunits that together constitute the 20S proteasome and the subsequent validation of 20S proteasome enzymatic activity in the exosomes suggested that the therapeutic activity of MSC exosomes could be partly attributed to the presence of 20S proteasome. 20S proteasome is responsible for the degradation of about 90% of all intracellular oxidatively damaged proteins[40] and reduced proteasomal activity has been postulated to be a contributing factor in the pathogenesis of aging-related neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease[41,42] or cardiovascular disease[43-45].

The biochemical potential of MSC exosomes to hydrolyze AMP to adenosine by CD73 and to subsequently induce phosphorylation of AKT and ERK1/2 through the adenosine receptor illustrated the capacity of MSC exosomes to hydrolyze AMP released by distressed cells and stimulated survival signaling pathways. A recent position paper from the European Society of Cardiology highlighted the activation of adenosine receptor and phosphorylation of the pro-survival kinases such as PI3 kinase-AKT and ERK1/2 as possibly having a role in the limiting reperfusion injury[46]. Therefore, the activation of adenosine receptor which has been shown to be cardioprotective[46,47]may also be a molecular mechanism mediating the amelioration of reperfusion injury by exosomes.

The inhibition of complement-mediated lysis of red blood cells by CD59 on exosomes represents yet another candidate mechanism for the cardioprotective effect of the exosome. Complement activation is a known mediator of ischemia/reperfusion injury in tissues such as intestines, heart and kidney, and its attenuation or inhibition has been shown to ameliorate tissue injury[48-50].

In summary, our interrogation and biochemical validation of the exosome proteome have uncovered a diverse range biochemical and cellular activities, and identified several candidate pathways for the cardioprotective effect of the exosome. Further validation studies in appropriate animal models will be required to determine if one or more of these candidate pathways contributed to the efficacy of MSC exosome in reducing reperfusion injury in the treatment of acute myocardial injury[51]. The multitude of biochemical potentials in MSC exosomes provides for the possibility of simultaneously targeting more than one mediator of tissue injury and potentially inducing a therapeutic synergy similar to that observed in combination drug therapy. This possibility of being able to target multiple mediators of injury is further enhanced by the use of enzymes to drive these targeting activities. Since enzyme activities are dictated by their microenvironment e.g. substrate concentration or pH, the enzyme-based therapeutic activities of exosomes could be activated or attenuated in proportion to the severity of disease-precipitating microenvironment. Consequently, the efficacy of exosome-based therapeutics could be highly responsive to and yet limited by the disease precipitating micro-environment. Together, these features could render exosome-based therapeutics intrinsically safer and more efficacious.

Example 19. Results - Activation of TLRs by MSC Exosomes

MSC exosomes were first assessed for their potential to activate TLRs using two cell lines derived from THP-1, a human acute monocytic leukemia cell line known to express most of the human TLRs. The first line is THP1-XBlue with a stably transfected secreted embryonic alkaline phosphatase (SEAP) reporter gene under the transcriptional control of NF-kB promoter. The second line is THP1-XBlue-defMYD that is deficient in MyD88 activity such that the activation of THP1-XBlue and not THP1-XBlue-defMYD indicates the presence of TLR ligands. At 0.1 μg/ml, MSC exosomes activated THP1-XBlue to the same extent as 0.01 μg/ml LPS and this activation unlike that of LPS was not abolished by polymyoxin B, ruling out LPS contamination in the MSC exosome preparation. Both MSC exosomes and LPS failed to activate THP1-XBlue-defMYD indicating that the activation of THP1-XBlue was mediated solely by activation of some of theTLRs present on THP-1 cells (Figure 6).

Since MSC exosomes contain putative ligands for TLR2 and TLR4, they were tested for the potential to activate these receptors using HEK reporter cell lines that are stably transfected with either human TLR2 or TLR4, and a SEAP reporter system (Figure 7 and Figure 8). Although MSC exosomes contained endogenous ligands for both TLR2 and TLR4, only TLR4 was activated at 0.1 μg/ml exosomes. There were some small but significant ($p<0.01$) activation of TLR2 at 1.0 μg/ml exosomes indicating that the capacity for TLR2 activation by MSC exosomes was at least 10 times lower. As TLR4 activation by exosome was not abolished by polymyoxin B, this ruled out LPS contamination in the MSC exosome preparation. Therefore MSC exosomes can activate TLR4 but not TLR2.

Example 20. MSC Exosome has TLR Ligands - Method

The presence of TLR ligands on MSC exosomes was determined using two cell lines derived from THP-1, a human acute monocytic leukemia cell line known to express most of the human TLRs. The first line is THP1-XBlue with a stably transfected secreted embryonic alkaline phosphatase (SEAP) reporter gene under the transcriptional control of NF-kB promoter. The second line is THP1-XBlue-defMYD that is deficient in MyD88 activity. 105 cells/well were seeded in a 96-well plate and incubated for 24 hours with 10 ηg/ml LPS (E. coli 026:B6, Sigma) or 0.1 μg/ml MSC derived exosome (MSC-Exo) with or without polymyoxin B, an antibiotic that binds and inactivates LPS. SEAP level was assayed using a Quanti-Blue (InvivoGen), a fluorescent substrate for alkaline phosphatase.

The results are shown in Figure 7.

Example 21. MSC Exosome does not Activate TLR2 - Methods

The TLR2 activation potential of MSC exosome was assessed using a commercially available HEK293 cell line that has been stably co-transfected with human TLR2, MD2 and CD14 and an optimized secreted embryonic alkaline phosphatase (SEAP) reporter gene under the control of the NF-κB promoter. This cell line, HEK-Blue-hTLR2 (Invitrogen) was seeded in a 96-well plate at 104 cells/well and incubated for 24 hours with 10 μg/ml lipoteichoic acid (LTA, Staphylococcus aureus, Sigma) or 0.1, 0.5 and 1.0 μg/ml MSC derived exosome (MSC-Exo). SEAP level was assayed using a Quanti-Blue (InvivoGen), a fluorescent substrate for alkaline phosphatase.

The results are shown in Figure 8.

Example 22. MSC Exosome Activates TLR4 – Methods

The TLR4 activation potential of MSC exosome was assessed using a commercially available HEK293 cell line that has been stably co-transfected with human TLR4, MD2 and CD14 and an optimized secreted embryonic alkaline phosphatase (SEAP) reporter gene under the control of the NF-κB promoter. This cell line, HEK-Blue-hTLR4 (Invitrogen) was seeded in a 96-well plate at 104 cells/well and incubated for 24 hours with 10 ηg/ml LPS (E. coli 026:B6, Sigma) or 0.1 μg/ml MSC derived exosome (MSC-Exo) with or without polymyoxin B, an antibiotic that binds and inactivates LPS. SEAP level was assayed using a Quanti-Blue (InvivoGen), a fluorescent substrate for alkaline phosphatase.

The results are shown in Figure 9.

Example 23. MSC Exosomes Induce Production of Pro- and Anti-inflammatory Cytokines by THP-1 Cells

Methods

THP-1 cells were seeded into 24-well culture plates at a concentration of 1×106 cells/ml, stimulated with 10 ηg/ml LPS or 0.1 μg/ml MSC-Exo for 0.5, 1, 3, 6, 12, 24, 48, 72, 96, and 120 hours. The cells were harvested and analysed for cytokine production by real-time quantitative RT-PCR. Total RNA was extracted using the RNeasy Mini kit (QIAGEN) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (ABI P/N.

4368813). PCR was performed on a StepOnePlus™ Real-Time PCR Systems (ABI P/N. 4376592) at a total volume of 20 µl in the presence of 10 µl of 2 × Fast SYBR® Green Master Mix (ABI, P/N 4385612) reaction buffer, 1 µl of cDNA, and 1 µl Oligonucleotide primers with an initial denaturation step at 95° C for 10 min followed by 40 cycles of denaturation at 95° C for 3 s, annealing and elongation at 60° C for 30 s. The primers used were as follows: IL-1β (FW: 5′ -CCTGTCCTGCGTGTTGAAAGA-3′ ; RV: 5′ -GGGAACTGGGCAGACTCAAA-3′), TNF-α (FW: 5′ -CCCCAGGGACCTCTCTCTAATC-3′ ; RV: 5′ -GGTTTGCTACAACATGGGCTACA-3′), IL-6 (FW: 5′ -TCGAGCCCACCGGGAACGAA-3′ ; RV: 5′ -GCAACTGGACCGAAGGCGCT-3′), IL-12p40 (FW: 5′ -CATGGTGGATGCCGTTCA-3′ ; RV: 5′ -ACCTCCACCTGCCGAGAAT-3′), IL-10 (FW: 5′ -GTGATGCCCCAAGCTGAGA-3′ ; RV: 5′ -CACGGCCTTGCTCTTGTTTT-3′), TGF-β1 (FW: 5′ -CAGCAACAATTCCTGGCGATA-3′ ; RV: 5′ -AAGGCGAAAGCCCTCAATTT-3′), GAPDH (FW: 5′ -GTCTTCACCACCATGGAGAAGGCT-3′ ; RV: 5′ -CATGCCAGTGAGCTTCCCGTTCA-3′). For each sample, mRNA content was normalized to that of human GAPDH mRNA and is illustrated as n-fold expression in comparison with untreated controls. Each sample was tested in triplicate. Data were analyzed using the comparative ΔCT method and Applied Biosystems StepOne software Version 2.0.1, according to the manufacturer's instructions.

Results

Effect of MSC exosomes on cytokine production by THP-1 cells.

Since TLRs are a major class of receptors that regulate activation of the innate immunity and MSC exosomes can activate TLRs in human monocytic cells, we determined if this activation leads to production of cytokines (Figure 10).

MSC exosomes induced a biphasic production of pro- and anti-inflammatory cytokines that peaked at 3 and 72-96 hours, respectively.

Example 24. Modulation of Adaptive Immunity by MSC Exosome

The activation of TLRs and induction of pro- and anti-inflammatory cytokines by MSC exosome suggest that MSC exosomes could modulate adaptive immunity. Specifically, we examine if MSC exosomes have any effects on Treg or Th17 differentiation. Treg refers to a subpopulation of T cells known as Regulatory T cells or suppressor T cells[12]. They downregulate the immune system and maintain tolerance to self-antigens. These cells have been used in immunotherapy for immune diseases such as allergy and asthma, diabetes, transplant rejection etc 13 14. Th17 cells are CD4+ memory T cells and are defined by their signature secretion of IL-17 cytokines. They are thought to be central to the pathogenesis of autoimmune disease[15].

To test the effects of MSC exosomes on differentiation of CD4+ mouse T cells into either Treg or Th17 cells, CD4+ mouse cells were incubated with MSC exosomes. As positive controls, CD4+ mouse cells were induced to differentiate into Treg cells by TGF-β1 and into Th17 cells by IL-6 & TGF-β1 either exerted no effects on the differentiation of CD4+ mouse T cells into either CD4+CD25+Foxp3+ Treg or Th17 cells (Figure 11).

Example 25. Inducing Differentiation of CD4+ Mouse T cells into Either Treg (a) or Th17 cells (b)

Murine splenocytes were prepared from 6 to 8-week-old BALB/C mice as previously described[16]. Briefly, mouse spleens were removed, minced and strained. Erythrocytes were then lysed. CD4+ cells were harvested using a FITC-conjugated Rat anti-mouse CD4 antibody with the EasySep FITC Selection Kit (StemCell Technologies) and the purity was verified by flow cytometry to be >95%. Purified $CD4^+$ T cells were activated using anti-CD3 mAb and 5 μg/ml anti-CD28 mAb.

a) For Treg differentiation: the culture medium was supplemented with 10 ηg/ml rhTGF-β1 or 0.1 μg/ml MSC-Exo. After 6 days, the number of Treg cells defined as CD4+, CD25+ and mouse Foxp3+ cells were determined by FACS using standard antibody staining techniques.

b) For Th17 differentiation, the culture medium was supplemented with 20 ηg/ml rIL-6 & 1 ηg/ml rhTGF-β1 or 0.1 μg/ml MSC-Exo. After 6 days, the number of Th17 cells

defined as CD4+, IL17a+ cells were determined by FACS using standard antibody staining techniques.

Treg and Th17 cells in a and b were normalized to the untreated cells.

Example 26. Modulation of Adaptive Immunity by MSC Exosome

Since MSC exosomes exert a profound activation effect on THP-1 cells and innate immunity plays a central role in modulating adaptive immune response , we hypothesized that exosome could modulate adaptive immunity via its effects on innate immunity. To test this hypothesis, we repeated the experiment described in Figure 11 with some modifications. During differentiation of the CD4+ T cells to either Treg or TH17 cells, the T cells were incubated with THP-1 cells that had been exposed to exosomes for 6 days (Figure 12). As positive controls, the T cells were incubated with TGF-β1 or rIL-6+ TGF-β1, respectively. THP-1 cells that had been exposed to MSC exosomes for 48 hours induced T cell differentiation into Tregs but not TH17 cells, suggesting that MSC exosomes could induce an immunosuppressive but not a pro-inflammatory T cell response.

Example 27. Modulating Adaptive Immunity via Innate Immunity

$CD4^+$ mouse T cells were purified and activated as described in Figure 11.

a) For Treg differentiation: the culture medium was supplemented with 10 ηg/ml rhTGF-β1, THP-1 cells or THP-1 cells exposed to 0.1 μg/ml MSC exosomes for 48 hours. The THP-1 cells added at at a ratio of 1 THP-1 to 1000 $CD4^+$ mouse T cells. After 6 days, the number of Treg cells defined as CD4+, CD25+ and mouse Foxp3+ cells were determined by FACS using standard antibody staining techniques.

b) For Th17 differentiation, the culture medium was supplemented with 20 ηg/ml rIL-6 & 1 ηg/ml rhTGF-β1 or or THP-1 cells exposed to 0.1 μg/ml MSC exosomes for 48 hours. The THP-1 cells added at at a ratio of 1 THP-1 to 1000 $CD4^+$ mouse T cells. After 6 days, the number of Th17 cells defined as CD4+, IL17a+ cells were determined by FACS using standard antibody staining techniques.

Treg and Th17 cells in a and b were normalized to the untreated cells.

Example 28. MSC Exosome Concanavalin A-activated Lymphocyte Proliferation Assay

MSC exosomes may be assessed by their ability to inhibit proliferation of Concanavalin A-activated lymphocytes. See Figure 13.

Mouse splenocytes were isolated and labeled with carboxyfluorescein succinimidyl ester (CFSE). The CFSE-labeled splenocytes were incubated with different concentration of MSC exosomes (0.1, 0.5, 1 and 4 μg/ml) in the presence or absence of 5 μg/ml Concanavalin A (ConA) for 3 days.

MSC exosomes were prepared as previously described (Lai et al, below). The number of fluorescent cells for each treatment group was quantitated by FACS. Data were normalized to the untreated control and presented as mean (± SD) of triplicate samples.

MSC-Exo did not affect proliferation of splenocytes ($p > 0.05$) and 4.0 μg/ml MSC-Exo significantly inhibited ConA-stimulated splenocytic proliferation (* $p < 0.01$).

Reference is made to R.C. Lai, F. Arslan, M.M. Lee, N.S. Sze, A. Choo, T.S. Chen, M. Salto-Tellez, L. Timmers, C.N. Lee, R.M. El Oakley, G. Pasterkamp, D.P. de Kleijn, S.K. Lim, Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury, Stem Cell Res, 4 (2010) 214-222.

Example 29. Conclusions (for Examples 19 to 28)

MSC exosomes can activate pro- and anti-inflammatory innate immune responses but can induce suppressive adaptive immune responses only indirectly via the innate immune system. They do not activate inflammatory adaptive immune responses directly or indirectly.

Therefore MSC exosomes could be used to enhance tissue repair and inhibit disease progression in autoimmune diseases such as GVHD, Crohn's disease, SLE, Diabetes Type 1, Multiple Sclerosis, ALS .

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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. 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.

CLAIMS

1. A method of detecting a therapeutic exosome, the method comprising detecting an activity of an exosome selected from the group consisting of: (a) immunodulatory activity; (b) complement inhibition activity; (c) proteasome activity; (d) glycolytic enzyme activity; (e) anti-oxidative activity; (f) extracellular matrix (ECM) modifying activity; (g) NT5E (CD73) ecto-5'-ectonucleotidase activity; (h) ion homeostasis activity; and (i) chaperone activity.

2. A method according to Claim 1, in which the activity comprises immunodulatory activity, the immunomodulatory activity being detected by detecting activity of a protein set out in Table D10.

3. A method according to Claim 1, in which the activity comprises complement inhibition activity, the complement inhibition activity being detected by detecting activity of a protein set out in Table D1.

4. A method according to Claim 1, in which the activity comprises proteasome activity, the proteasome activity being detected by detecting activity of one or more, preferably all, proteins set out in Table D2.

5. A method according to Claim 1, in which the activity comprises glycolytic enzyme activity, the glycolytic enzyme activity being detected by detecting activity of one or more, preferably all, proteins set out in Table D3.

6. A method according to Claim 1, in which the activity comprises anti-oxidative activity, the anti-oxidative activity being detected by detecting activity of one or more, preferably all, proteins set out in Table D4.

7. A method according to Claim 1, in which the activity comprises extracellular matrix (ECM) modifying activity, the extracellular matrix (ECM) modifying activity being detected by detecting activity of one or more, preferably all, proteins set out in Table D5.

8. A method according to Claim 1, in which the activity comprises NT5E (CD73) ecto-5'-ectonucleotidase activity, the NT5E (CD73) ecto-5'-ectonucleotidase activity being detected by detecting activity of one or more, preferably all, proteins set out in Table D6.

9. A method according to Claim 1, in which the activity comprises ion homeostasis activity, the ion homeostasis activity being detected by detecting activity of one or more, preferably all, proteins set out in Table D7.

10. A method according to Claim 1, in which the activity comprises chaperone activity, the chaperone activity being detected by detecting activity of one or more, preferably all, proteins set out in Table D8.

11. A method according to any preceding claim, in which if an activity is detected, the exosome is likely to be capable of: (i) inhibiting complement mediated cell lysis; or (ii) reducing infarct size for example as assayed in a mouse or pig model of myocardial ischemia and reperfusion injury, or which is capable of reducing oxidative stress for example as assayed in an *in vitro* assay of hydrogen peroxide (H_2O_2)-induced cell death, or both (i) and (ii).

12. A method according to Claim 11, in which the therapeutic activity comprises cardioprotective activity.

13. A method according to Claim 11, in which the therapeutic activity is against one or more diseases 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, 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.

14. A method comprising the steps of: (a) isolating an exosome from a mesenchymal stem cell conditioned medium (MSC-CM), optionally comprising separating the exosome from other components based on molecular weight, size, shape, composition or biological activity, and (b) detecting an activity set out in Claim 1 in the isolated exosome, preferably by detecting the activity of one or more proteins set out in any of Claims 2 to 9.

15. A method of producing an exosome, the method comprising: (a) obtaining a mesenchymal stem cell conditioned medium (MSC-CM); (b) concentrating the mesenchymal

stem cell conditioned medium, for example by ultrafiltration over a >1000 kDa membrane; (c) subjecting the concentrated mesenchymal stem cell conditioned medium to size exclusion chromatography, such as using a a TSK Guard column SWXL, 6 x 40 mm or a TSK gel G4000 SWXL, 7.8 x 300 mm column; (d) selecting UV absorbant fractions, for example, at 220 nm, that exhibit dynamic light scattering, such as detected by a quasi-elastic light scattering (QELS); in which step (d) for example comprises collecting fractions which elute with a retention time of 11-13 minutes, such as 12 minutes; and detecting an activity set out in Claim 1 in the exosome, preferably by detecting the activity of one or more proteins set out in any of Claims 2 to 9.

16. A method substantially as hereinbefore described with reference to and as shown in Figures 1 to 13 of the accompanying drawings.