CHARACTERIZATION OF THE SECRETION OF MESENCHYMAL STEM CELLS AND ITS RELEVANCE TO CARDIOPROTECTION

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2011

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(B.Eng. (Hons.)), NTU

A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

NUS Graduate School for Integrative Sciences and Engineering

NATIONAL UNIVERSITY OF SINGAPORE

2011

ACKNOWLEDGEMENTS

I would like to acknowledge and extend my heartfelt gratitude to the following persons who have made the completion of this PhD thesis possible:

My supervisor, Associate Professor Lim Sai Kiang, for her encouragement, guidance and unreserved support from start to finish.

Members of my thesis advisory committee, Dr. Alan Colman, Professor Shazib Pervaiz and Associate Professor Lu Jinhua, for their useful suggestion, assistant and guidance.

Dr. Dominique de Kleijn and Dr. Fatih Arslan, our collaborators in the Laboratory of Experimental Cardiology, Utrecht Medical Center, for their help in animal model study, guidance and useful discussion.

Dr. Andre Choo, Dr. Lee May May, Mdm. Jayanthi Padmanabhan, Mr. Jeremy Lee, Mr. Hoi Kong Meng and Mr. Eddy Tan, our collaborators in Bioprocessing Technology Institute, for their help in the preparation of conditioned medium, purification of exosomes and technical guidance.

Dr. Yin Yijun, Dr. Chen Tiansheng, Dr. Zhang Bin, Mr. Teh Bao Ju, Mr. Tan Soon Sim, Mr. Ronne Yeo Wee Ye, my colleagues in Institute of Medical Biology, for their help, encouragement, useful discussion and company throughout my stay in the lab.

Elsevier Limited and Future Medicine Limited, for the permission to reproduce the manuscripts in the thesis.

Most especially to my wife-to-be Ms. Liow Sing Shy, for her love, support and encouragement. Thank you!

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SUMMARY

Acute myocardial infarction (AMI), which is caused by occlusion of coronary artery, results in myocardial infarction and this may eventually contribute to the development of heart failure. Ironically, reperfusion therapy, which restores blood flow and significantly limits ischemic injury, causes reperfusion injury and contributes to the final infarct size. Amelioration of reperfusion injury will therefore improve the efficacy of reperfusion therapy. However, there is still no effective treatment to limit reperfusion injury, and this is contributing to a growing epidemic of heart failure. Recent developments have indicated that secretion of mesenchymal stem cells (MSCs) can reduce reperfusion injury. However, the cardioprotective factor in the secretion and underlying mechanism of its cardioprotection remains to be elucidated.

To identify the active component in MSC secretion, 0.2 μM filtered culture medium conditioned by human embryonic stem cell-derived MSCs was filtered sequentially through filters with decreasing pore sizes. Only the >1000 kDa fraction reduced infarct size in a mouse MI/R injury model. This physically limited the size of cardioprotective factor to 100-220 ηm and the candidate factor to exosome. Electron microscopy showed the presence of 100 ηm particles in the conditioned medium. Further analysis revealed the presence of co-immunoprecipitating exosome-associated proteins and the co-sedimentation of these proteins with membrane lipids after ultracentrifugation. These proteins were determined to have an exosome-like flotation density of 1.10-1.16 μg/ml by sucrose gradient centrifugation. These exosomes could be purified by size

exclusion on HPLC and this purified exosome significantly reduced infarct size in the same mouse model.

To assess if the secretion of cardioprotective exosome was restricted to hESC-derived MSCs, we derived 5 MSCs cultures from various tissues of 3 first-trimester aborted fetuses. These MSCs were highly expandable, displayed typical MSC surface antigen and gene expression profile, and possessed the MSC tri-lineage differentiation potential. Like hESC-MSCs, they produced exosomes that were cardioprotective in mouse MI/R injury model. Therefore, production of cardioprotective exosomes was not restricted to hESC-MSCs but was common to all MSCs.

To understand the cardioprotective mechanism of MSC exosome, the biochemical potential of exosome *in vitro* and *in vivo* was assessed. Proteomic profiling of exosome identified 866 proteins that together had the potential to drive diverse biological processes. Several of these processes had the potential to reduce injury during reperfusion including enhancing glycolysis, inhibiting the formation of membrane attack complex, reducing oxidative stress and activating pro-survival kinases. Consistent with the *in vitro* data, exosome treatment in mouse model promoted pro-survival signaling, enhanced ATP production and redox balance. These probably contributed to the reduced infarct size and preserved cardiac function and geometry that observed in the exosomes treated group.

In summary, we identified exosome as the cardioprotective component in MSCs secretion. We further demonstrated that secretion of cardioprotective exosomes was not restricted to hESC-MSCs and suggested potential mechanisms underlying this cardioprotection. These findings not only redefined the paracrine mechanism

of MSCs, more importantly they might lead to the development of adjunctive reperfusion therapy.

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LIST OF ABBREVIATIONS

AAR	Area at risk
ACN	Acetonitrile
AMC	7-amino-4 methylcoumarin
AMI	Acute myocardial infarction
ANX	Annexins
CM	Conditioned medium
DLS	Dynamic light scatter
EDP	End-diastolic pressure
EDV	End-diastolic volume
EEFA1	Eukaryotic translation elongation factor 1A1
EF	Ejection fraction
ENO	Enolase
ESV	End-systolic volume
FA	Formic acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
hESC-MSCs	Human ESC-derived MSCs
HPLC	High performance liquid chromatography
IS	Infarct size
ITS	Insulin, transferrin, and selenoprotein
ki	Kidney
lb	Limb
LCA	Left coronary artery
li	Liver

LV	Left ventricular
MAC	Membrane attack complex
MFGE8	Milk fat globule EGF factor 8 protein
MI	Myocardial infarction
MI/R	Myocardial ischemia/reperfusion
MSCs	Mesenchymal stem cells
MSN	Moesin
MVBs	Multivesicular bodies
MWCO	Molecular weight cut off
NCM	Nonconditioned medium
NT5E	Ecto-5'-ectonucleotidase
PANTHER	Protein analysis through Evolutionary Relationships
PBS	Phosphate buffered saline
PGK	Phosphoglycerate kinase
PGM	Phosphoglucomutase
PK	Pyruvate kinase
PKm2	Pyruvate kinase m2 isoform
PMSA1-7	Proteasome alpha subunit 1-7
PMSB1-7	Proteasome beta subunit 1-7
PV	Pressure-volume
RDX	Radixin
SRBCs	Sheep red blood cells
SWT	Systolic wall thickening
TFF	Tangential flow filtration
U	Unit

WBC	White blood cell
WT	Wall thickness

AUTHOR CONTRIBUTIONS

This PhD thesis was completed as part of collaboration with different laboratories. The author played a major role in the experimental design, execution and analysis. The mouse MI/R injury model and Langendorff model were done in collaboration with Professor Dominique de Kleijn at the Laboratory of Experimental Cardiology, University Medical Center, Utrecht. The mass spectrometry analysis of conditioned medium and exosomes were performed by Assistant Professor Newman Sze from Nanyang Technological University. The preparation of conditioned medium, purification of the exosome and transmission electron microscope analysis of conditioned medium were done by Dr. Andre Choo, Bioprocessing at the Technology Institute. For all these collaborations, the author participated in the collection and analysis of the experimental data.

INTRODUCTION

This introduction are adapted with modifications from a published review article "Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease"[1] *of which I am the first author.*

Myocardial Ischemia/Reperfusion Injury

Acute myocardial infarction (AMI), commonly known as heart attack occurs during a sudden obstruction of blood supply to part of the heart by vulnerable atherosclerotic plaque rupture[2]. AMI causes substantial irreversible cell death if left untreated for a substantial period of time[3]. Based on estimates by World Health Organization, 7.2 million people died from AMI in 2004, representing 12% of all global deaths. It is projected that by 2030, almost 10 million people will die from AMI, a 38% increase in 25 year[4]. In Singapore, AMI accounted for 19.2% of all deaths in 2009[5], which was the number two cause of death.

Reperfusion therapy or the restoration of blood flow by percutaneous coronary intervention (PCI), thrombolytic therapy or bypass surgery is currently the mainstay of treatment for AMI and is responsible for the significant reduction in AMI mortality[6]. It was shown that the mortality rate of AMI in Germany reduced from 16.2% in 1994 to 9.9% in 2002 in tandem with the increasing use of reperfusion therapy[6]. The efficacy of reperfusion therapy has led to increasing survival of patients with severe AMI who would not otherwise survive. Despite adequate reperfusion, however, most patients still suffer irreversible myocardial cell loss. Ironically, reperfusion itself is an important contributor to irreversible myocardial cell loss due to a phenomenon referred to as reperfusion injury[7]. Based

on studies in animal models of AMI, reperfusion injury contributed up to 50% of the final infarct size[7]. Amelioration of reperfusion injury and subsequent reduction of myocardial infarct size will dramatically improve patient prognosis. More importantly, by reducing reperfusion injury, the progression of AMI to heart failure that is highly dependent on infarct size[8-13] might be reduced, thus relieving the phenomenon of the ever-growing epidemic of heart failures[14-16].

It was recognized by Jennings et al. as early as 1960 that reperfusion of severely ischemic tissue causes lethal injury[17]. They observed significant morphological alteration in ischemic canine myocardium after the onset of reperfusion. These include cardiomyocyte swelling, mitochondrial clarification, amorphous/flocculent densities representing calcium phosphate deposits, hypercontracture and loss of sarcomere organization. It was believed that several abrupt biochemical and metabolic changes during reperfusion causes lethal reperfusion injury. These include the generation of reactive oxygen species (ROS)[18,19], intracellular Ca^{2+} overload[20], the rapid restoration of physiologic pH[21] and inflammation[22]. The complex interaction of each biochemical and metabolic changes, together mediates cardiomyocyte death through apoptosis, necrosis, inflammation and hypercontracture[7]. But the very existence of lethal reperfusion injury was actively debated[23]. It became more widely accepted only when infarct size was shown to be reduced by interventions applied at the onset of reperfusion[8]. These interventions including postconditioning which involved several cycles of brief mechanically interrupted-reperfusion applied at the onset of reperfusion or pharmacological agents applied before the onset of reperfusion have demonstrated some protection against reperfusion injury in animals or small clinical trials in terms of reducing infarct size and/or improving heart function[8]. The obvious

implication of these findings is that adjunctive therapies at the onset of reperfusion might salvage more myocardium at risk. Unfortunately, over the past 30 years, most of the agents failed to reproduce these beneficial effects in large-scale clinical trial and none has been translated into clinical practice[24-27]. This have led to speculations that reducing reperfusion injury may not be tractable to pharmaceutical interventions[28].

Mesenchymal Stem Cells In The Treatment Of Acute Myocardial Infarction

With the emergence of stem cells as potential regenerative medicine, attempts to use stem cells to reduce infarct size and enhance cardiac function in animal models and patients have increased exponentially. To date, stem cell therapy for the heart accounts for one third of the publications in the regenerative medicine field[29]. The rationale for the use of stem cells to repair cardiac tissues was based on the hypothesis that these cells could differentiate into cardiomyocytes and supporting cell types to replace cells lost during MI/R injury, and achieve cardiac repair[30]. Among stem cells currently being tested in clinical trials for the heart, MSCs are the most widely used stem cells. Part of the reasons is their easy availability in accessible tissues such as bone marrow aspirate, fat tissue[31] and their large capacity for ex vivo expansion[32]. MSCs are also known to have immunosuppressive properties[33]. Therefore another attractive advantage is that they could be used in allogeneic transplantation which is very practical in clinic. Besides, they are also reported to have highly plastic differentiation potential that included not only adipogenesis, osteogenesis and chondrogenesis[34-39], but also endothelial and cardiovascular differentiation[40], neurogenic differentiation[41-43], and neovascular differentiation[44-46].

MSCs transplantation in most AMI animal models generally resulted in reduced infarct size, improved left ventricular ejection fraction, increased vascular density, and myocardial perfusion[47-51]. In a recent phase I randomized double blind placebo-controlled dose-escalation clinical trial, single infusion of allogeneic MSCs in patients with AMI was documented to be safe with some provisional indications that the MSC infusion improved outcome with regard to cardiac arrhythmias, pulmonary function, left ventricular function, and symptomatic global assessment[52].

Despite numerous studies on the transplantation of MSCs in patients and animal models, insight into the mechanistic issues underlying the effect of MSC transplantation remains vague. An often-cited hypothesis is that transplanted MSCs differentiate into cardiomyocyte and supporting cell types to repair cardiac tissues. However, contrary to this differentiation hypothesis, most transplanted MSCs are entrapped in the lungs and the capillary beds of tissues other than the heart[53,54]. And depending on the method of infusion, 6% or less of the transplanted MSCs persist in the heart two weeks after transplantation[55]. In further contradiction, transplanted MSCs were observed to differentiate inefficiently into cardiomyocytes[56] while ventricular function was rapidly restored less than 72 h after transplantation[57]. All these observations are physically and temporally incompatible with the differentiation hypothesis and have thus prompted an alternative hypothesis that the transplanted MSCs mediate their therapeutic effect through secretion of paracrine factors that promote survival and tissue repair[58].

Paracrine Secretion of MSCs

Paracrine secretion of MSCs was reported more than 15 years ago when Haynesworth et al. reported that MSCs synthesize and secrete a broad spectrum of growth factors and cytokines such as VEGF, FGF, MCP-1, HGF, IGF-I, SDF-1 thrombopoietin[59-63] that exert effects on cells in their vicinity [64]. Paracrine secretion have been postulated to promote arteriogenesis[61]; support the stem cell crypt in the intestine[65]; protect against ischemic renal[59,60] and limb tissue injury[62]; support and maintain hematopoiesis[63]; support the formation of megakaryocytes and proplatelets[66]; and promote breast cancer metastasis[67]. Many of these factors such as VEGF, HGF, bFGF were also found to exert beneficial effects on the heart, including neovascularization[68], attenuation of ventricular wall thinning[50] and increased angiogenesis[69,70].

In 2005, Gnecchi et al. showed that intramyocardial injection of either culture medium conditioned by MSCs overexpressing the Akt gene (Akt-MSCs) or the Akt-MSCs reduced infarct size in a rodent model of AMI to the same extent. This provided the first direct evidence that cellular secretion alone could be cardioprotective[57,71]. Again, in 2008, Timmers et al. showed that culture medium conditioned by hESC-MSCs significantly reduced infarct size by approximately 50% in a pig and mouse model of MI/R injury when administered intravenously in a single bolus just before reperfusion[72]. These observations represent a very important step forward in our understanding of the cardioprotective mechanism of MSC-based therapy in AMI. It clearly demonstrated that cardiac repair could be achieved without the actual participation of the cells themselves but by simply administering their secretion. These discoveries explained the fast acting effect of MSCs after transplantation and explained why the low efficiency of engraftment

and differentiation did not affect the efficacy of the MSC transplantation. More importantly, these findings could potentially facilitate the translation of cell-free secretion as an adjunctive therapy to reperfusion therapy if the active cardioprotective factor of these paracrine secretions could be identified.

Thesis

The specific aims of this PhD project were to identify the active cardioprotective factor of the MSCs secretion and to elucidate the mechanisms of the cardioprotection. The findings from this project have been either published in peer-reviewed papers or are in manuscripts under peer review for publication. The papers and manuscripts are attached in the following chapter. I am also a co-author of 4 publications[73-76] where I contributed my expertise in exosome biology. These publications are in areas that are not directly relevant to my thesis.

Four papers in the following chapters described the work leading to discoveries that exosome is the cardioprotective factor in the MSC secretion, secretion of cardioprotective exosome is a property of MSCs, exosome carries a cargo that has diverse biochemical and cellular potential and the exosome elicits cellular responses that are known to be cardioprotective and are consistent with its biochemical cargo.

In the first paper, "Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury", we addressed the question "What is the active cardioprotective factor of the MSCs secretion?" By filtering the secretion sequentially through filters with decreasing pore sizes, fractions containing molecules within different molecular weight ranges were generated. We found

that only >1000 kDa 0.2μM filtered fraction reduced infarct size. This finding limited the physical size of cardioprotective factor to 100-220 ηm, which is much larger than the typical paracrine mediators that usually consist of growth factors, cytokines and chemokines[77]. Under the transmission electron microscope, we observed ~100 ηm diameter particles in the secretion. Based on the size range and morphology of these particles and current research literature we postulated that the likely candidate was a secreted phospholipid vesicle known as exosome.

We next investigated whether exosomes are present in the secretion. We first did a proteomic analysis of MSC secretion using mass spectrometry and antibody array to check if the secretion contained proteins that are commonly found in exosomes such as CD9, CD81 and Alix[78]. 738 proteins were detected and these included most of the reported exosomes-associated protein. The presence of some of exosomes-associated proteins was confirmed by Western blot analysis. Furthermore, we observed exosome-associated proteins, CD9 and Alix, co-immunoprecipitated with another exosome-associated proteins, CD81, suggesting that these proteins were in a single complex. As exosomes are routinely purified by ultracentrifugation, we checked if we could precipitate CD9 by ultracentrifuging the secretion. The result showed that CD9 could be precipitated. At the same time, we also observed enrichment of major plasma membrane component such as cholesterol, sphingomyelin and phosphotidylcholine in the ultracentrifugation pellet. We further checked if the flotation densities of CD9 and CD81 fall within the typical density range of exosome, which is 1.10-1.18 g/ml. The results showed that they both floated in the density range of exosome and pretreatment with a detergent-based cell lysis buffer decreased the apparent flotation densities of CD9 and CD81 to that of proteins in a similar molecular

weight range. By limited trypsinization, we also showed that CD9, a membrane-bound protein, was partially susceptible to trypsin digestion, and this partial susceptibility of CD9 was detergent-sensitive. This was consistent with its localization in a lipid membrane. In summary, these observations suggested the existence of exosomes in the secretion.

To prove the existence of exosome in the secretion, we tried to purify exosomes from the secretion by size exclusion on a HPLC. The first 8 eluted fractions (F1 to F8, based on the absorbance profile at 220 nm) from HPLC were collected. Only F1 to F4 contained proteins as shown by silver staining. Proteins were distributed among F2, F3, and F4 fractions according to the principle of size-exclusion fractionation that larger proteins were eluted first followed by smaller proteins. Proteins in F2 were generally larger than those in F3 which in turn were larger than those in F4. In contrast, proteins in the F1 fraction had a MW distribution that spanned the entire MW spectrum of F2, F3, and F4. Dynamic light scattering analysis showed that F1 contained homogeneously sized particles with a hydrodynamic radius of 55-65 ηm. Western blot analysis showed that CD9 was present exclusively in the F1 and had a flotation density in the range of exosome, i.e. 1.10-1.18 g/ml. These features of the F1 fraction, i.e., the proteins with a wide spectrum of MW, exclusive presence of CD9 and homogenously sized particles were consistent with presence of exosome and indicated that exosomes were successfully purified from the secretion by HPLC fractionation. When 0.4 μg of F1 proteins were administered to a mouse model of MI/R injury 5 min prior to reperfusion, it reduced infarct size to the same extent as 3 μg secretion proteins. In summary, we had identified exosome as the cardioprotective component in MSC secretion.

In the second paper, “Derivation and characterization of human fetal MSCs: an alternative cell source for large-scale production of cardioprotective microparticles”, we assessed if cardioprotective exosome was secreted by MSCs in general. Five MSC cultures were derived from limb, kidney and liver tissues of 3 first-trimester aborted fetuses. These fetal tissue-derived MSCs have a stable karyotype and similar telomerase activities to hESC-MSCs. They are highly expandable, each line has the potential to generate at least $10^{16\text{-}19}$ cells or $10^{7–10}$ doses of cardioprotective secretion for a pig model of MI/R injury. They displayed a typical MSC surface antigen profile, but unlike previously described fetal MSCs, they did not express pluripotency-associated markers such as Oct4, Nanog or Tra1-60. They have the potential to differentiate into adipocytes, osteocytes and chondrocytes *in vitro*. Global gene expression analysis by microarray revealed a typical MSC gene expression profile that was highly correlated among the five fetal MSC cultures and with that of hESC-MSCs. Most importantly, like hESC-MSCs, they produced exosomes that were cardioprotective in a mouse model of MI/R injury. Together we demonstrated that fetal tissues-derived MSCs also produced cardioprotective exosome and that the secretion of protective exosomes was not an exclusive characteristic of hESC-MSCs but possibly a universal property of all MSCs.

In the third paper, “Characterizing the biological potency of MSC exosome by cellular and biochemical validation of its proteome”, we assessed the biochemical potential of exosome *in vitro* to identify candidate mechanisms for

cardioprotective effect of hESC-MSCs. We profiled the proteome of MSC exosome to identify 866 proteins. These proteins could be functionally clustered into 32 over-represented biological processes. Together, these suggested exosomes had a potential to drive a diverse spectrum of cellular and biochemical activities. To evaluate and verify this potential, we selected proteins for which assays to assess either their biochemical and/or cellular activities were 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 that degrade oxidized protein.

All five enzymes (GAPDH, PGK, PGM, ENO, PKm2) in the ATP generating stage of the glycolysis were present in the exosome proteome. In addition, PFKFB3 a powerful allosteric activator of phosphofructokinase, which catalyzes the commitment to glycolysis[79], was shown to be present in the phosphorylated form. This predicted that exposure of cells to exosome could result in increased glycolytic flux in the cells. Consistent with the prediction, exosomes significantly increased ATP level in oligomycin-treated cells. Another group of proteins, PMSA1-7 and PMSB1-7, which form the 20S proteasome, were also detected in our exosome proteome. The presence of all seven α- and all seven β-subunits of the 20S core particle suggest that MSC exosomes contained intact 20S proteasome complexes and therefore potentially possessed 20S proteasome enzymatic activity.

Consistent with this, MSC exosome was able to degrade short fluorogenic peptides and this degradation was inhibited by lactacystin, a specific proteasome inhibitor.

Besides these 2 groups of proteins, CD73, an enzyme that converts AMP into adenosine, was also found in exosome proteome. This suggested that exosomes might have the potential to induce adenosine-mediated signaling. Consistent with this hypothesis, we demonstrated exosomes could hydrolyze AMP to adenosine by CD73 and subsequently induced phosphorylation of AKT and ERK1/2 in a serum starvation cell model. This phosphorylation of ATK and ERK1/2 could be abolished by theophylline, a non-selective adenosine receptor antagonist that antagonized A1, A2A, A2B, and A3 receptors[80]. In addition, we also verified the functional ability of another important protein detected in exosome, CD59, an inhibitor of the formation of membrane attack complex (MAC). We showed that MSC exosomes were able to inhibit complement-mediated lysis of sheep red blood cells. This inhibition was abolished when a CD59 blocking antibody was used to pre-treat the exosome, showing that CD59 of exosomes was directly involved in the inhibition of complement lysis. All together, in this paper, our interrogation and biochemical validation of the exosome proteome have uncovered a diverse range of biochemical and cellular activities and identified several candidate biological processes 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 AMI.

In the fourth paper, “Exosomes target multiple mediators to reduce cardiac injury”, we described both therapeutic and mechanistic actions of MSC exosomes in an animal model MI/R injury. First, we studied the mode of action of exosome cardioprotection by asking whether exosomes exert their therapeutic effect via blood cells or via direct interaction with myocardial cells. Exosome treatment in *ex vivo* MI/R experiments by the Langendorff setup reduced infarct size to the same extent as *in vivo*. This suggested that exosomes directly targeted myocardial cells to reduce MI/R injury without involving circulating cells. We further showed that vigorous agitation to disrupt exosome abolished its cardioprotective effect. This demonstration highlighted the importance of intact lipid membrane in mediating exosome cardioprotection.

We then examined the cardiac performance of exosome-treated heart. Functional and geometry assessment of left ventricle by MRI measurement showed significant preservation of both end-diastolic and end-systolic volume, improved ejection fraction, decreased thinning of the infarct area during scar maturation and improved systolic thickening of the infarcted area in the exosome-treated group. These observations were consistent with the infarct size reduction seen after exosome treatment. By invasive pressure-volume loop recording, we also observed higher contractility and relaxation in exosome treated mice 28 days after infarction, which is consistent with the consequences of reduced dilation and improved systolic performance. Besides functional improvement, we also observed attenuation of inflammation including reduced neutrophil infiltration and reduced white blood cell count after MI/R injury in exosome treated mice. These are likely secondary to the reduced cardiac injury after exosome treatment.

Having established the functional improvement in exosomes treated heart after MI/R injury, we explored potential mechanisms of this therapeutic effect. We selectively evaluated the potential mechanisms that we previously proposed in our exosome biochemical study paper to confirm whether they are valid in the *in vivo* MI/R injury model. We first explored the possibility that exosome reduced infarct size by activating survival pathways, especially PI3K/AKT pathway. Exosome treatment induced AKT and GSK3 phosphorylation within 1-hour after reperfusion. However, ERK1/2 phosphorylation was not altered in exosome treated group. These suggested that exosomes specifically target AKT and GSK3 pathway to induce pro-survival effects. At the same time, c-JNK, a known activator of pro-apoptotic was significantly dephosphorylated. In addition, we demonstrated that ADP/ATP and NAD^+/NADH ratio in the area at risk of exosome-treated mice were significantly lower in 30 minutes after reperfusion compared with the saline-treated control. These observations were consistent with our previous *in vitro* biochemical and cellular validations. Together, these findings highlighted the fast acting effect of exosomes and suggested that activating survival pathway, enhancing ATP production and correcting redox balance through glycolysis might be the potential cardioprotective mechanisms of exosome. In conclusion, this study showed the therapeutic action of exosome and suggested potential mechanisms of exosomes in ameliorating reperfusion injury.

PAPER ONE

Exosome Secreted By MSC Reduces Myocardial Ischemia/Reperfusion Injury

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Stem Cell Research (2010) 4, 214–222

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REGULAR ARTICLE

Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury

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Received 29 July 2009; received in revised form 22 December 2009; accepted 22 December 2009

Abstract Human ESC-derived mesenchymal stem cell (MSC)-conditioned medium (CM) was previously shown to mediate cardioprotection during myocardial ischemia/reperfusion injury through large complexes of 50–100 nm. Here we show that these MSCs secreted 50- to 100-nm particles. These particles could be visualized by electron microscopy and were shown to be phospholipid vesicles consisting of cholesterol, sphingomyelin, and phosphatidylcholine. They contained coimmunoprecipitating exosome-associated proteins, e.g., CD81, CD9, and Alix. These particles were purified as a homogeneous population of particles with a hydrodynamic radius of 55–65 nm by size-exclusion fractionation on a HPLC. Together these observations indicated that these particles are exosomes. These purified exosomes reduced infarct size in a mouse model of myocardial ischemia/reperfusion injury. Therefore, MSC mediated its cardioprotective paracrine effect by secreting exosomes. This novel role of exosomes highlights a new perspective into intercellular mediation of tissue injury and repair, and engenders novel approaches to the development of biologics for tissue repair.

Abbreviations: AMI, acute myocardial Infarction; CM, conditioned medium; MI/R, myocardial ischemia/reperfusion; MSCs, mesenchymal stem cells; MWCO, molecular weight cut off; NCM, nonconditioned medium.

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doi:10.1016/j.scr.2009.12.003

Introduction

Mesenchymal stem cells (MSCs) derived from adult bone marrow have emerged as one of the most promising stem cell types for treating cardiovascular disease (Pittenger and Martin, 2004). Although the therapeutic effect of MSCs has been attributed to their differentiation into reparative or replacement cell types (e.g., cardiomyocytes, endothelial cells, and vascular smooth cells) (Minguell and Erices, 2006; Zimmet and Hare, 2005), it remains to be established if the number of differentiated cell types generated is therapeutically relevant. Recent reports have suggested that some of these reparative effects are mediated by paracrine factors secreted by MSCs (Caplan and Dennis, 2006a; Gnecchi et al., 2005, 2006; Schafer and Northoff, 2008). In support of this paracrine hypothesis, many studies have observed that MSCs secrete cytokines, chemokines, and growth factors that could potentially repair injured cardiac tissue mainly through cardiac and vascular tissue growth and regeneration (Caplan and Dennis, 2006b; Liu and Hwang, 2005). This paracrine hypothesis could potentially provide for a non-cell-based alternative for using MSCs in treatment of cardiovascular disease (Pittenger and Martin, 2004). Non-cell-based therapies as opposed to cell-based therapies are generally easier to manufacture and are safer as they are nonviable.

We have previously performed an unbiased proteomic analysis of a chemically defined medium conditioned by highly expandable human ESC-derived MSC cultures (Lian et al., 2007; Sze et al., 2007). We identified >200 proteins in the secretion of these MSCs (Sze et al., 2007). Computational analysis of the secretome predicted that collectively, the secretome has the potential to repair injured tissue such as in myocardial ischemia/reperfusion (MI/R) injury (Sze et al., 2007). MI/R injury refers to cell death and functional deterioration that occurs during reperfusion therapy to restore blood flow and salvage cardiomyocytes at risk of dying from ischemia in an acute MI (AMI) (Cannon et al., 2000; Saraste et al., 1997). Therefore, the effectiveness of reperfusion therapy can be greatly enhanced by preventing reperfusion injury for which there is currently no treatment (Knight, 2007). We tested the computational prediction of tissue salvage during reperfusion injury in a pig and mouse models of MI/R injury. An intravenous bolus administration of MSC-CM just before reperfusion substantially reduced infarct size in both pig and mouse models of MI/R injury by ~60 and ~50%, respectively (Timmers et al., 2008). There was also a significant preservation of cardiac function and reduction of oxidative stress as early as 4 h after reperfusion (Timmers et al., 2008). However, the active component in the secretion and the mechanism by which it mediates this fast-acting effect on MI/R injury have not been elucidated.

It is obvious that the immediacy of this protective effect precludes the relatively lengthy process of gene transcription and tissue regeneration as part of the mechanism. Also, many of the secreted proteins are membrane and intracellular proteins, and are not known to cross plasma membranes readily. This suggests that if these proteins mediate the cardioprotective effect, the mechanism underlying the therapeutic effect of MSC secretion must involve a vehicle that facilitates crossing of membranes, thus representing a radical shift from our present understanding of MSC paracrine secretion which is limited to extracellular signaling by cytokines, chemokines, and growth factors. To better understand the cardioprotective paracrine effects of MSCs, we then systematically fractionated the MSC-CM using membranes with different molecular weight cut off (MWCO). Based on these fractionations, we demonstrated that the cardioprotective activity was in a >1000-kDa MW fraction (Timmers et al., 2008). This suggested that the cardioprotective effect was mediated by large complexes with a diameter of 50–100 nm.

Here we demonstrate that these large complexes are exosomes. By improving our proteomic analysis, we extended our previously reported list of 201 secreted proteins to 739 proteins and observed the presence of many exosome-associated proteins. Some of these proteins were in detergent-sensitive complexes. These proteins can be sedimented by ultracentrifugation together with the membrane phospholipids. Size-exclusion fractionation by HPLC and dynamic light scattering analysis revealed the presence of a population of particles with a hydrodynamic radius (R_h) of 55–65 nm. More importantly, this HPLC fraction reduced infarct size in a mouse model of MI/R injury.

Results

Cardioprotective secretion contains exosome-associated proteins that form multiprotein complexes

To identify the active component, we had previously fractionated the CM by ultrafiltration through membranes with different MWCO. It was shown that CM filtered through a membrane with MWCO of 1000 kDa was not protective in a mouse model of MI/R injury (Timmers et al., 2008). However, CM concentrated by 125 times against a similar membrane was protective. We observed that after filtration through filters with a MWCO smaller than 0.2 mm such as 100, 300, 500, or 1000 kDa, the filtered CM was not cardioprotective (Fig. 1A). In contrast, CM concentrated against a 1000-kDa membrane (Timmers et al., 2008) or a 100-kDa membrane to retain particles >1000 or 100 kDa, respectively, was cardioprotective (Fig. 1). These observations suggested that the active fraction consisted of large complexes of >1000 kDa or had a predicted diameter of 50–100 nm. Consistent with this, visualization of the CM by electron microscopy revealed the presence of spherical structures with a diameter of 50–100 nm and the morphology of a lipid vesicle (Fig. 1B). Based on this size range and morphology, we postulated that the likely candidate was a secreted phospholipid vesicle known as exosome (Fevrier and Raposo, 2004; Keller et al., 2006).

To test this, we first determined if the CM contained the subset of proteins that are commonly found in exosomes such as CD9, CD81, and Alix (Olver and Vidal, 2007). These proteins were not present in our previous proteomic profiling of the secretion (Sze et al., 2007). By making modifications to our proteomics methodology, we extended our list of proteins found in the MSC secretion from 201 to 739 proteins (Supplementary Table 1). The computationally predicted biological activities of this proteome suggested that the secretion will have significant biological effects on cardiac

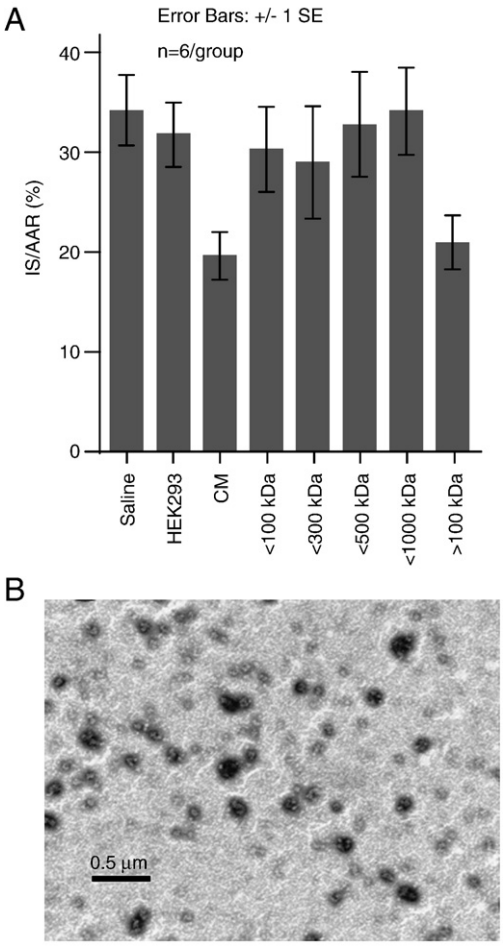

Figure 1 Cardioprotective properties of CM fractions. (A) Saline, HEK293 CM, or different preparations of hESC-MSC CM were administered to a mouse model of MI/R injury as described under Materials and methods. The <1000, <500, <300, and <100 kDa represented CM filtered sequentially with membranes that had MWCO of 1000, 500, 300, and 100 kDa, respectively. The >100 kDa represented CM concentrated 50 times against a TFF membrane with MWCO of 100 kDa. The infarct size (IS) was expressed as a fraction of the area at risk (AAR) in the left ventricle. (B) Transmission electron microscopic picture of CM; scale bar represents 500 nm.

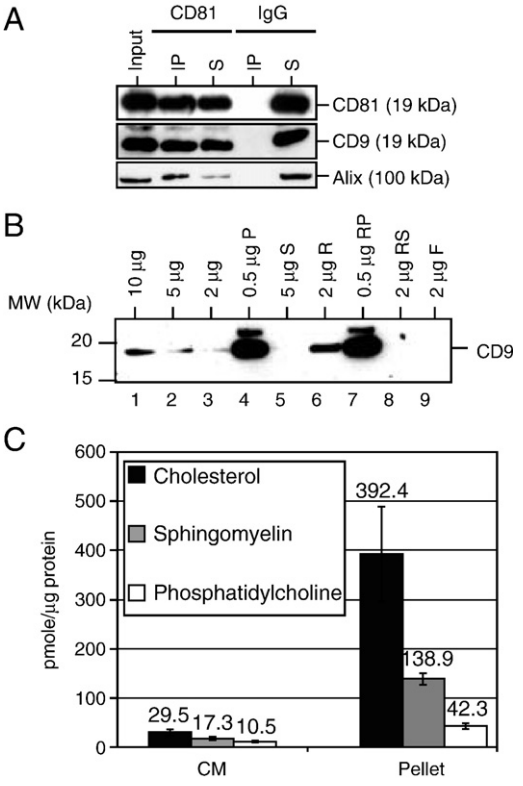

Figure 2 Presence of large lipid complexes in CM. (A) Coimmunoprecipitation of CD81, CD9, and Alix. After immunoprecipitation of hESC-MSC CM with anti-CD81 or mouse IgG, the immunoprecipitate (IP) and supernatant (S) were analyzed by Western blot hybridization using antibody against CD9 and Alix. (B) Size fractionation by ultrafiltration and ultracentrifugation. CM was concentrated 5X using a membrane with MWCO of 500 kDa. The retentate and the unfiltered CM were then ultracentrifuged at 200 000 *g* for 2 h. The supernatant and the pellet were analyzed by Western blotting for the presence of CD9. Lanes 1–3: Different protein amount of CM. Lanes 4 and 5: The pellet (P) and supernatant (S) after ultracentrifugation of unfiltered CM. Lane 6: Retentate (R) after filtration of CM through a membrane with MWCO of 500 kDa. Lanes 7 and 8: The pellet (RP) and supernatant (RS) after ultracentrifugation of retentate. Lane 9: Filtrate (F) after filtration of CM through a membrane with MWCO of 500 kDa. (C) Amount of cholesterol, spingomyelin, and phosphatidylcholine in CM and in the pellet after 200 000 *g* ultracentrifugation of the CM was assayed and quantitated as picomole per microgram protein.

tissue injury and repair (Fig. S1). The subsets of exosome-associated proteins CD9, CD81, and Alix were confirmed to be present in the secretion by Western blot analysis (lane 1, Fig. 2A). The MW of CD9 and CD81 was the expected 25 and 22–26 kDa, respectively. Consistent with our hypothesis that the large complexes are exosomes, we observed that CD9 and Alix coimmunoprecipitated with CD81, suggesting that these proteins were in a single complex (Fig. 2A).

A 24-kDa CD9 sediments at 200 000 *g* and is retained by membrane with 500-kDa MWCO

As exosomes are routinely purified by ultracentrifugation (Thery et al., 2006), we next determined if CD9 was associated with a large complex that can be precipitated by ultracentrifugation. The CM was first fractionated through a membrane (MWCO = 500 kDa) into a >500-kDa retentate fraction and a <500-kDa filtrate fraction followed by ultracentrifugation of both fractions. The 24-kDa CD9 was found in the >500-kDa retentate fraction and could be precipitated by ultracentrifugation (Fig. 2B). CD9 was not detected in the <500-kDa filtrate fraction. Consistent with our exosome hypothesis, major plasma membrane phospholipids such as cholesterol, sphingomyelin, and phosphatidylcholine were also precipitated by ultracentrifugation at 200 000 *g* for 2 h as evidenced by their enrichment in the precipitate (Fig. 2C).

Proteins in the CM are associated with phospholipid membrane

As exosomes are phospholipid vesicles, they are known to have a typical density range of 1.10 to 1.18 g ml^{-1} that could be resolved on sucrose gradients (Raposo et al., 1996; Thery et al., 2006). We therefore postulated that the flotation densities of the putative exosome-associated proteins would be different before and after release from such vesicles by a detergent-based buffer. Therefore, CM or CM pretreated with a detergent-based lysis buffer was fractionated on a sucrose density gradient by equilibrium ultracentrifugation. The fractions were analyzed for the distribution of CD9 and CD81. Both CD9 and CD81 which coimmunoprecipitated (Fig. 2A) had a similar flotation density that was heavier than that expected of proteins in their MW range (Fig. 3). Pretreatment with a detergent-based cell lysis buffer decreased the apparent flotation densities of CD9 and CD81 to that of proteins in a similar MW range (Fig. 3). Our observations demonstrated that the detergent-sensitive flotation densities of proteins were consistent with their location in lipid vesicles.

Exosomal proteins are either membrane bound or encapsulated

As many of the secreted proteins in the CM are known membrane or cytosolic proteins, we investigated if these proteins in the CM were also membrane bound or localized within the lumen of the putative exosomes by limited trypsinization. Membrane-bound proteins would expected to be partially resistant whereas luminal proteins are expected to be resistant to trypsinization. Treatment with

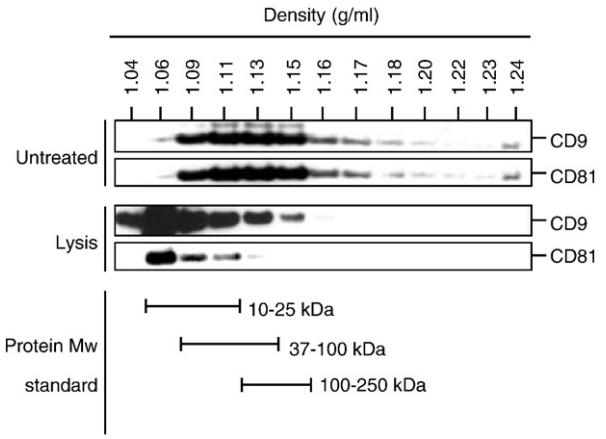

Figure 3 Protein analysis of CM fractionated on a sucrose gradient density. CM or CM pretreated with lysis buffer was loaded on a sucrose density gradient prepared by layering 14 sucrose solutions of concentrations from 22.8 to 60% (w/v) in a SW60Ti centrifuge tube and then ultracentrifuged for 16.5 h at 200 000 *g*, 4 °C, in a SW60Ti rotor. The gradients were removed from the top and the density of each fraction was calculated by weighing a fixed volume of each fraction. The fractions were analyzed by Western blot analysis for CD9 and CD81 in CM (upper panel) and pretreated CM (lower panel). The distribution of a protein standard molecular weight marker set after fractionation in a similar gradient is denoted at the bottom of the figure.

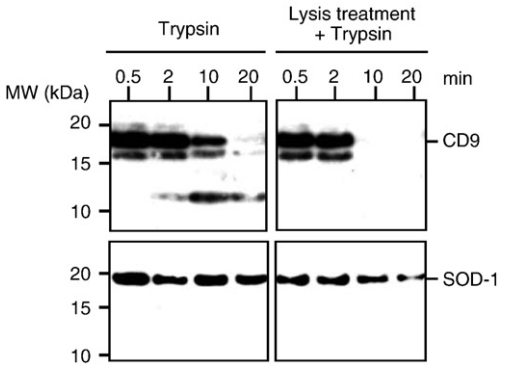

Figure 4 Trypsinization of CM. CM treated with either PBS or lysis buffer was digested with trypsin and an aliquot was removed at 0.5, 2, 10, and 20 min. A trypsin inhibitor, PMSF, was then added to terminate the trypsinization reaction and the aliquots were analyzed for the presence of CD9 and SOD-1 by Western blot hybridization.

a detergent-based lysis buffer would abrogate this resistance. As expected, CD9, a membrane-bound protein was susceptible to trypsin digestion and generated two detectable tryptic peptide intermediates (Fig. 4). In contrast, SOD-1, a cytosolic protein was resistant to trypsin digestion. Pretreatment of CM with a detergent-based cell lysis buffer abolished the resistance of CD9 and SOD-1 to trypsin digestion. The detergent-sensitive partial susceptibility of CD9 and resistance of SOD-1 to trypsin digestion were consistent with their localization in a lipid membrane and lumen of an exosome, respectively.

Purification of a homogeneous population of exosomes by HPLC fractionation

To demonstrate directly that the active cardioprotective component in the secretion is an exosome, CM and nonconditioned medium (NCM) were first fractionated by size exclusion on a HPLC column (Fig. 5A).The eluent was monitored by absorbance at 220 nm and then examined by dynamic light scattering which has a hydrodynamic radius (R_h) detection range of 1 to 1000 nm. The first four eluting fractions in CM (F1–F4) were not present in the NCM and therefore represented secretion from the hESC-MSCs. F1, the fastest eluting fraction with a retention time of 12 min, represented the fraction containing the largest particles in the CM. The particles in F1 were sufficiently homogeneous in size such that they could be determined by dynamic light scattering to have a hydrodynamic radius (R_h) of 55–65 nm. All other peaks contained particles that were too heterogeneous in size to be estimated by dynamic light scattering. F1 contained 4% of total protein input but contained ~50% of the CD9 in the input (Fig. 5B). Proteins were distributed among F2, F3, and F4 fractions according to the principle of size-exclusion fractionation such that larger proteins were eluted first in F2 followed by the smaller proteins in F3 and the smallest in F4 (Fig. 5C). In contrast, proteins in the F1 fraction had a MW distribution that spanned the entire MW spectrum of F2, F3, and F4 (Fig. 5C). The proteins in the F1 fraction despite having a MW range of 20 to 250 kDa

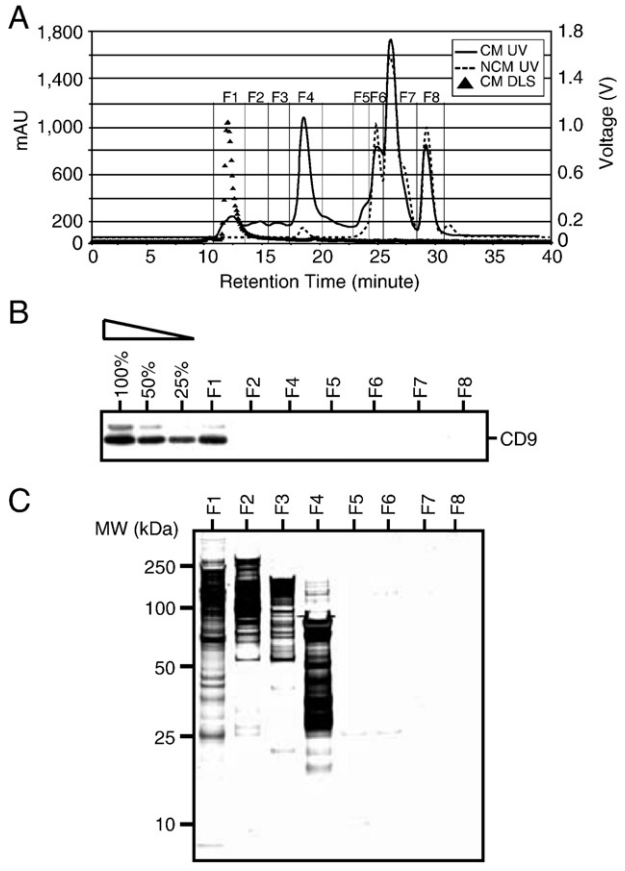

Figure 5 HPLC fractionation of CM. (A) HPLC fractionation and dynamic light scattering of CM and NCM. CM and NCM were fractionated on a HPLC using a BioSep S4000, 7.8 mm×30 cm column. The components in CM or NCM were eluted with 20 mM phosphate buffer with 150 mM NaCl at pH 7.2. The elution mode was isocratic and the run time was 40 min. The eluent was monitored for UV absorbance at 220 nm. Each eluting peak was then analyzed by light scattering and signals as measured in voltage are represented by solid triangles. The eluted fractions, F1 to F8, were collected, their volumes were adjusted to 50% of the input volume of CM, and an equal volume of each fraction was analyzed for (B) the presence of CD9 by Western blot hybridization. Lanes 1–3 were CM loaded at 2X, 1X, or 0.5X of the volume loaded used for each of the fractions, F1 to F8 (lanes 4–11), and therefore represented the equivalent of 100, 50, and 25% input CM. (C) Equal volumes of F1–F8 were separated on a SDS-PAGE and then stained with silver.

sedimented at a similar flotation density of 1.11–1.16 g/ml (Fig. 6B) that was similar to that of CD9 in the CM (Fig. 3). These features of the F1 fraction, i.e., the presence of proteins with a wide spectrum of MW sizes and identical flotation density, the exclusive presence of CD9, and a homogeneous size, indicated that a homogeneous exosome population was purified from the CM by HPLC fractionation. When 0.4 μg of F1 protein was administered to a mouse model of MI/R injury 5 min prior to reperfusion, the F1 fraction reduced infarct size to the same extent as 3 μg CM protein (Fig. 7A). All animals in this study had the same degree of endangered myocardium, as illustrated by similar area at risk within the left ventricle (Fig. 7B).

Paracrine effect was mediated through heart tissues

For elucidating the mechanism of this paracrine effect, an important prerequisite is the identification of the target tissues. Here we determine that the paracrine effect on MR/I injury was a heart autonomous effect and was independent of circulating cells including immune cells. Using an *ex vivo* mouse Langendorff heart model of ischemia/reperfusion injury, we observed that conditioned medium reduced relative infarct size to the same extent as in a mouse model (Fig. 8).

Discussion

The trophic effects of MSC transplantation on ameliorating the deleterious consequences of myocardial ischemia have been implicated in several studies (Caplan and Dennis, 2006a). Transplantation of MSCs into ischemic myocardium has been shown to induce several tissue responses such as an increased

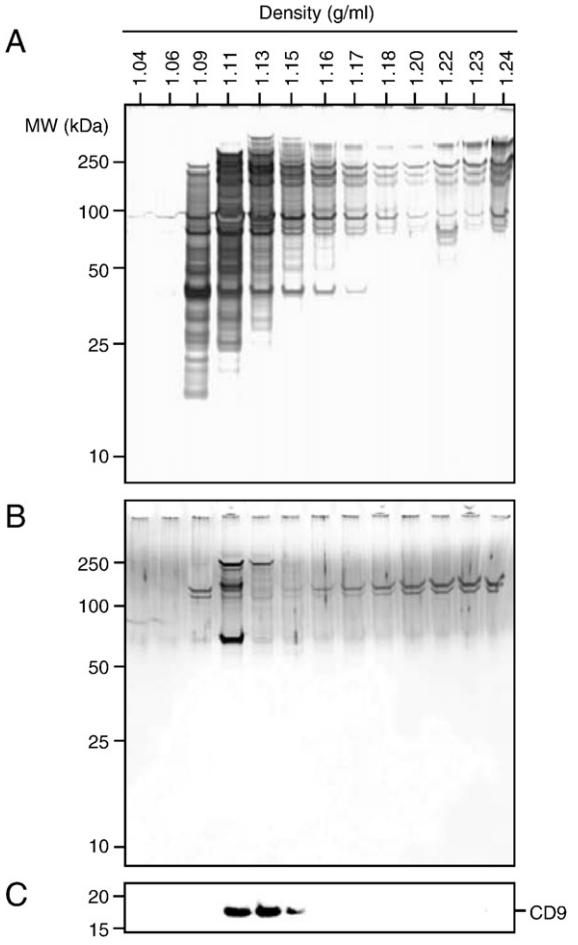

Figure 6 Flotation densities of proteins in CM and HPLC-purified F1 fraction were determined by fractionating CM and F1 onto a sucrose gradient density as described above. The 13 fractions for (A) CM and (B) F1 were separated on a SDS-PAGE and then stained with silver. (C) To evaluate the distribution of smaller proteins, the F1 was also assayed for CD9 (20 kDa) by Western blot hybridization. Proteins in the >1.20 g/ml density fractions were denatured proteins.

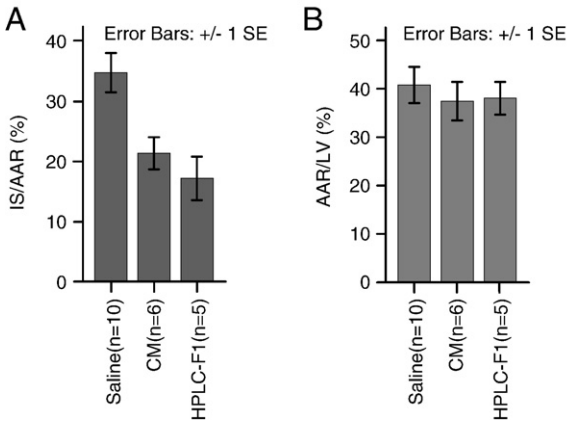

Figure 7 Cardioprotective exosomes. A 0.4 μg F1 protein was administered intravenously to a mouse model of MI/R injury 5 min before reperfusion. Infarct sizes (IS) as a percentage of the area at risk (AAR) on treatment with saline ($n=10$), conditioned medium from hESC-MSCs ($n=6$), and HPLC fraction ($n=5$) were measured. Saline treatment resulted in 34.5±3.3% infarction. CM treatment resulted in 21.2±2.6% infarction ($P=0.022$ compared to saline) and F1 fraction treatment resulted in 17.0±3.6% infarction ($P=0.004$ compared to saline). (B) AAR as a percentage of the left ventricle (LV), showing the amount of endangered myocardium after MI/R injury. All animals were affected to the same extent by the operative procedure, resulting in 39.1±2.2% of AAR among the groups. Each bar represents mean±SEM.

production of angiogenic factors and decreased apoptosis (Tang et al., 2005). It was postulated that these responses were better explained by secretion of paracrine factors than by differentiation of MSCs. In this context, MSCs were shown to secrete many growth factors and cytokines that have

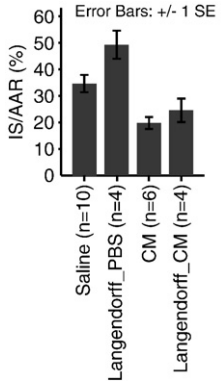

Figure 8 Secretion reduced myocardial ischemia-reperfusion injury *ex vivo*. Perfusion buffer containing 3.5 μg/ml CM was used to perfuse mouse heart in an *ex vivo* mouse Langendorff heart model of MI/R injury 5 min before reperfusion. Infarct sizes (IS) as a percentage of the area at risk (AAR) on treatment with PBS ($n=4$) and CM ($n=4$) were measured after 3 h reperfusion. Langendorff_PBS treatment resulted in 49.3±5.3% infarction. Langendorff_CM treatment resulted in 24.6±4.4% infarction ($P<0.001$ compared to Langendorff_PBS). As a reference for comparison, the *in vivo* effects of saline and CM on IS/AAR as described in Fig. 7 are also included.

effects on cells in their vicinity. To date, many of these studies have focused exclusively on proteins that are known to be secreted. These proteins generally included cytokines, chemokines, and other growth factors (Caplan and Dennis, 2006a). However, our unbiased proteomic profiling of proteins in the secretion of MSCs revealed an abundance of membrane and cytosolic proteins (Sze et al., 2007). This suggests that the trophic effects of MSCs may not be mediated by soluble growth factors and cytokines alone. This was underscored by our observation that the cardioprotective effects of CM were mediated by 50- to 100-nm complexes of >1000 kDa (Timmers et al., 2008) and not small soluble proteins.

Based on the size of the complex, we postulated that the cardioprotective complex in the CM was likely to be an exosome. Exosomes are formed from multivesicular bodies with a bilipid membrane (Fevrier and Raposo, 2004; Keller et al., 2006). They have a diameter of 40–100 nm and are known to be secreted by many cell types (Fevrier and Raposo, 2004; Keller et al., 2006). Electron microscopy confirmed that the CM contained lipid-like vesicles of about 50–100 nm in diameter. The functions of exosomes are not known but they are thought to be important in intercellular communications. Although exosomes are known to have a cell-type-specific protein composition, most carry a common subset of proteins that included CD9, CD81, Alix, TSP-1, SOD-1, and pyruvate kinase (Olver and Vidal, 2007). CD9 and CD81 are tetrapannin membrane proteins that are also localized in the membrane of exosomes. Consistent with the presence of exosomes, CM contained coimmunoprecipitating complexes of CD81, CD9, and Alix. Ultracentrifugation precipitated CD9 with phospholipids and cholesterol, suggesting that the CD81, CD9, and Alix complex was associated with a phospholipid vesicle. This was confirmed by the detergent-sensitive flotation densities of these proteins where we demonstrated that the flotation densities of these proteins in the CM were that of phospholipid vesicles and that detergent treatment which dissolved phospholipid membrane altered the flotation densities of the proteins. We further demonstrated that CD9 in the CM was a membrane-bound protein while SOD-1 was localized within a lipid vesicle by their respective partial and complete resistance to trypsin degradation and the abrogation of this resistance by detergent. Taken together, our observations demonstrated that exosomes with a diameter of 50–100 nm are present in the CM and are therefore the likely candidate for the cardioprotective component in the CM. This was confirmed when a HLPC-purified homogeneous population of particles that had an enrichment of CD9 and a R_h of 55–65 nm substantially reduced infarct size in a mouse model of MI/R injury at a reduced protein dosage equivalent to ~10% of the CM dosage. We further demonstrated using an *ex vivo* mouse Langendorff heart model of MI/R injury that this paracrine effect was a heart autonomous effect, and was independent of circulating cells, such as immune cells or platelets.

In summary, we have identified exosome as the cardioprotective component in MSC paracrine secretion. This involvement of exosomes represents a radical shift in our current understanding of the paracrine effect of MSC transplantation on tissue repair which hitherto has been limited to cytokine, chemokine, or growth factor-mediated extracellular signaling. It also highlights for the first time the role of exosome as mediator of tissue repair. As lipid vesicles,

exosomes represent an ideal vehicle to effect an immediate physiological response to repair and recover from injury through the rapid intracellular delivery of functional proteins. Recently, it was demonstrated that in addition to proteins, microvesicles have the potential to mediate intercellular transfer of genetic material (reviewed in Quesenberry and Aliotta, 2008). Several tumor cell types (Rosell et al., 2009; Taylor and Gercel-Taylor, 2008), peripheral blood cells (Hunter et al., 2008; Valadi et al., 2007), endothelial progenitor cells (Deregibus et al., 2007), and embryonic stem cells (Ratajczak et al., 2006) have been shown to secrete RNA-containing microvesicles. More importantly, these microvesicular RNA could be transferred to other cells and translated in the recipient cells (Deregibus et al., 2007; Ratajczak et al., 2006; Valadi et al., 2007). We also recently demonstrated that the MSC-derived exosomes described here also contained miRNAs and these miRNAs were predominantly in the precursor form (Chen et al., 2010). For reperfused ischemic myocardium, this feature of rapid initiation of cellular repair through the intracellular delivery of functional proteins and possibly RNA is particularly critical as the time window for therapeutic intervention is very narrow. We speculate that the involvement of exosomes in cardioprotection may represent a general function of exosomes in tissue repair. It is possible that different cell types produce exosomes that are specific for certain type of cells or injuries. If true, this novel tissue-repair function of exosomes could potentially engender new approaches to the development of biologics.

Materials and methods

Preparation of CM

The culture of HuES9.E1 cells and preparation of HuES9.E1 CM were performed as described previously (Lian et al., 2007; Sze et al., 2007). For the <100-, <300-, <500-, or <1000-kDa preparations in Fig. 1, the CM was first concentrated 25X by tangential flow filtration (TFF) using a membrane with a 10-kDa MWCO (Sartorius, Goettingen, Germany) and then filtered sequentially through membranes with MWCO of 1000 kDa (Sartorius), 500 kDa (Millipore, Billerica, MA), 300 kDa (Sartorius), and finally 100 kDa (Sartorius). All other CM and NCM used were concentrated 25X or 50X by TFF using a membrane with 10- or 100-kDa MWCO (Sartorius). The CM and NCM preparations were filtered with a 0.2-μm filter before storage or use.

Electron microscopy, antibody array assay, protein analysis

Electron microscopy, antibody array assay, and protein analysis were done using standard protocols; for details please refer to Supplementary Materials and Methods.

LC MS/MS analysis

Proteins in 2 ml of dialyzed CM or NCM were analysis by LC MS/MS using standard protocols with some modifications; for details please refer to Supplementary Materials and Methods.

Immunoprecipitation of exosome-associated proteins

Dynabead M-280 sheep anti-mouse IgG (Invitrogen Corporation, Carlsbad, CA) was washed using 0.1% BSA/PBS before incubation with mouse anti-human CD81 antibody for 2 h with gentle shaking at room temperature. The dynabeads were washed twice and incubated with CM with gentle shaking for 2 h at room temperature. The supernatant was then collected, and the dynabeads were gently washed twice before PBS was added. The supernatant and the dynabeads were denatured, resolved on 4–12% SDS-PAGE, and analyzed by Western blotting.

Sucrose gradient density equilibrium centrifugation

To generate the sucrose gradient density for centrifugation, 14 sucrose solutions with concentrations from 22.8 to 60% were prepared and layered sequentially in an ultracentrifuge tube (Beckman Coulter Inc., CA) starting with the most concentrated solution. CM was loaded on top before ultracentrifugation for 16.5 h at 200 000 *g*, 4 °C in a SW60Ti rotor (Beckman Coulter Inc.). After centrifugation, 13 fractions were collected starting from the top of the gradient. The densities of each were determined by weighing a fixed volume. For pretreatment with detergent-based lysis buffer (Cell Extraction Buffer, Biovision, Mountain View, CA), CM was incubated with an equal volume of the lysis buffer containing protease inhibitors (Halt Protease Inhibitor Cocktail, Thermo Fisher Scientific Inc., Waltham, MA) for 30 min at room temperature with gentle shaking. The protein concentration of CM was quantified using the NanoOrange Protein Quantification kit (Invitrogen Corporation) according to the manufacturer's instructions.

Sphingomyelin, phosphatidylcholine, and cholesterol assay

Cholesterol, sphingomyelin, and phosphatidylcholine concentration in CM and pellet from the ultracentrifugation of CM at 200 000 *g* for 2 h at 4 °C was determined using commercially available assay kits. Cholesterol was measured using the Amplex Red Cholesterol Assay kit (Invitrogen Corporation), sphingomyelin, and phosphatidylcholine were measured using the Sphingomyelin Assay Kit and Phosphatidylcholine Assay Kit (Cayman Chemical Company, Ann Arbor, MI) respectively.

Limited trypsinization of CM

CM was incubated with equal volumes of either PBS or lysis buffer (Cell Extraction Buffer, Biovision, Mountain View, CA) for 45 min at 4 °C with gentle shaking. Then 16 μl of 10× trypsin (Invitrogen Corporation) was added and incubated at 37 °C with gentle shaking. An aliquot was removed at 30 s, 1 min, 5 min, and 20 min, and 1 μl of a 100 mM trypsin inhibitor, PMSF (Sigma-Aldrich, St. Louis, MO), was added. The mixture was denatured and analyzed by Western blot analysis.

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, 6×40 mm and TSK gel G4000 SWXL, 7.8×300 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 (CA, USA) and were operated by the ASTRA software. For details please refer to Supplementary Materials and Methods.

MI and surgical procedure

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Pigs prepared by the Institute of Laboratory Animal Resources and with prior approval by the Animal Experimentation Committee of the Faculty of Medicine, Utrecht University, the Netherlands. The CM and the HPLC fraction 1 (F1) were tested in a mouse model of MI/R injury. MI was induced by 30 min left coronary artery (LCA) occlusion and subsequent reperfusion. Five minutes before reperfusion, mice were intravenously infused with 200 μl saline-diluted CM containing 3 μg protein or HPLC F1 containing 0.4 μg protein via the tail vein. Control animals were infused with 200 μl saline. After 24 h reperfusion, infarct size (IS) as a percentage of the area at risk (AAR) was assessed using Evans' blue dye injection and TTC staining as described previously (Arslan et al., 2010).

Mouse Langendorff heart model of ischemia/reperfusion injury

For the mouse Langendorff heart model of ischemia/reperfusion injury, mice were given heparin 50 IE subcutaneously. The suture was placed *in vivo* without placing the knot. Hereafter, the heart was excised and aortic root was canulated and perfused in the Langendorff setup. After 10 min recovery, the suture was tightened to induce ischemia for 30 min. Just 5 min prior to reperfusion, the perfusion buffer was changed for a second buffer containing 3.5 μg/ml MSC-CM. Reperfusion was allowed for 3 h before Evans' blue dye injection and TTC staining for infarct size assessment, as described previously (Arslan et al., 2009).

Acknowledgments

We gratefully acknowledge Kong Meng Hoi and Eddy Tan at the Bioprocessing Technology Institute (BTI) for helping in the purification of the exosomes and Jayanthi Padmanabhan and Jeremy Lee (BTI) for the preparation and concentration of the conditioned medium.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scr.2009.12.003.

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Supplementary Table 1
Alphabetical list of 739 unique gene products identified by LC-MS/MS and antibody array

02-Sep	BPNT1	COL5A3	FAM3C	HINT1	ITGB4BP	MYH9	PPIA	RPLP2	THBS2
07-Sep	BTD	COL6A1	FAM49B	HIST1H4	K-ALPHA-1	MYL6	PPIB	RPS10	THOP1
AARS	C14orf141	COL6A2	FAM62A	HIST1H4A	KPNB1	NAGK	PPP2R1A	RPS15A	THY1
ACAA2	C19orf10	COL6A3	FBLN1	HIST1H4B	KRT1	NANS	PPP2R4	RPS16	TIMP1
ACAT2	C1orf58	COL7A1	FBLN5	HIST1H4C	KRT14	NARS	PPP5C	RPS19	TIMP2
ACO1	C1orf78	COPA	FBN1	HIST1H4D	KRT2	NEDD8	PPP6C	RPS2	TIMP3
ACTB	C1QBP	COPG	FBN2	HIST1H4E	KRT27	NEFM	PRDX1	RPS20	TKT
ACTC1	C1R	COPS3	FDPS	HIST1H4F	KRT4	NIT2	PRDX2	RPS23	TLN1
ACTN1	C1S	COPS4	FGF16	HIST1H4H	KRT5	NME1	PRDX3	RPS3	TMOD2
ACTN2	C21orf33	COPS8	FGFRL1	HIST1H4I	KRT6L	NPC2	PRDX4	RPS4X	TMOD3
ACTN3	CALR	CORO1B	FH	HIST1H4J	KRT7	NPEPPS	PRDX5	RPS5	TNC
ACTN4	CAND1	CORO1C	FKBP10	HIST1H4K	KRT75	NPM1	PRDX6	RPS7	TNFRSF11B
ACTR1A	CAP1	COTL1	FKBP1A	HIST1H4L	KRT77	NQO1	PRG1	RPS8	TNFRSF12A
ACTR1B	CAP2	CRIP2	FKBP3	HIST2H2AA3	KRT9	NRP1	PRKACA	RPS9	TNFSF12
ACTR2	CAPG	CS	FLNA	HIST2H2AA4	KRTHB4	NRP2	PRKCSH	RPSA	TNPO1
ACTR3	CAPN1	CSE1L	FLNB	HIST2H4A	LAMA4	NT5E	PRNP	RSU1	TP53I3
ACTR3B	CAPN2	CSRP1	FLNC	HIST2H4B	LAMB1	NUCB1	PROCR	RTN4	TPI1
ADAM9	CAPZA1	CSRP2	FLRT2	HIST4H4	LAMC1	OLFML3	PROSC	S100A11	TPM1
ADSL	CAPZA2	CST3	FLT1	HLA-A	LANCL1	P4HA1	PRSS23	S100A16	TPM2
ADSS	CAPZB	CTGF	FN1	HLA-B	LAP3	P4HB	PRSS3	SARS	TPM3
AEBP1	CARS	CTHRC1	FSCN1	HMX1	LASP1	PABPC1	PSAP	SDC4	TPM4
AGA	CBR1	CTSB	FSTL1	HNRPA1	LDHA	PABPC4	PSAT1	SDCBP	TRAP1
AGRN	CBR3	CTSD	FSTL5	HNRPA1L-2	LDHAL6B	PAFAH1B1	PSMA1	SEC22B	TRHDE
AHCY	CCBL2	CTSZ	FTL	HNRPA2B1	LDHB	PAFAH1B2	PSMA2	SEC23A	TROVE2
AK1	CCDC19	CXCL1	G6PD	HNRPC	LEPRE1	PAFAH1B3	PSMA3	SEC31A	TSKU
AK2	CCL18	CXCL12	GALNT2	HNRPCL1	LGALS1	PAICS	PSMA6	SEMA3C	TUBA1A
AKR1A1	CCL2	CXCL16	GALNT5	HNRPD	LGALS3	PAM	PSMA7	SEMA7A	TUBA6
AKR1B1	CCL7	CXCL2	GANAB	HNRPDL	LGALS3BP	PAPPA	PSMB1	SERPINB1	TUBA8
ALCAM	CCN4	CXCL9	GAPDH	HNRPH2	LMNA	PARK7	PSMB2	SERPINB6	TUBB
ALDH2	CCR4	CYCS	GARS	HNRPK	LOC196463	PARP1	PSMB3	SERPINE1	TUBB2C
ALDH7A1	CCR5	D4ST1	GAS6	HNRPL	LOC283523	PARVA	PSMB4	SERPINE2	TUBB3
ALDOA	CCT2	DAG1	GBA	HNRPR	LOC347701	PCBP1	PSMB5	SERPINF1	TUBB4
ALDOC	CCT3	DCI	GBE1	HNRPU	LOC646821	PCBP2	PSMD11	SERPINH1	TUBB6
ANGPT4	CCT4	DCN	GDF1	HNT	LOC649125	PCDH18	PSMD13	SERPINI2	TUBB8
ANP32B	CCT5	DDAH2	GDF11	HSP90AB1	LOC653214	PCDHGB6	PSMD5	SFRP1	TWF1
ANXA1	CCT6A	DDB1	GDF15	HSP90B1	LOC654188	PCK2	PSMD6	SFRP4	TXN
ANXA2	CCT7	DDT	GDF3	HSPA1A	LOC728378	PCMT1	PSMD7	SH3BGRL3	TXNL5
ANXA5	CCT8	DDX17	GDF5	HSPA1B	LOXL2	PCNA	PSME1	SIL1	TXNRD1
ANXA6	CD109	DES	GDF8	HSPA1L	LRP1	PCOLCE	PSME2	SLC1A5	UBE1
AP1B1	CD248	DKK1	GDI1	HSPA4	LTA	PDCD6IP	PTBP1	SLC3A2	UBE2L3
AP1S1	CD44	DLD	GDI2	HSPA5	LTA4H	PDGFA	PTK7	SND1	UBE2N
AP2A1	CD59	DNAJC3	GLO1	HSPA6	LTB	PDGFC	PTPRCAP	SNRPD1	UBE2V1
AP2A2	CD81	DPP3	GLRX	HSPA8	LTB4DH	PDGFRB	PTX3	SNRPE	UBE3B
AP2B1	CD9	DPYSL2	GLT8D3	HSPB1	LTBP1	PDIA3	PURA	SOD1	UCHL1
AP3B1	CDC37	DPYSL3	GLUD1	HSPD1	LTBP2	PDIA4	PXDN	SPARC	UCHL3
APEX1	CDC42	DSTN	GM2A	HSPE1	LUM	PDIA6	PYCR1	SPOCK	UGDH
API5	CDH11	DYNLL1	GNPDA1	HSPG2	M6PRBP1	PDLIM1	PYGB	SPTAN1	UGP2
APOA1BP	CDH13	ECHS1	GNPNAT1	HSPH1	MACF1	PDLIM5	QARS	SPTBN1	UROD
APOE	CDH2	ECM1	GOT1	HTRA1	MADH4	PDLIM7	QPCT	SPTBN4	USP14

APP	CFL1	EEF1A1	GOT2	IDH1	MAP1B	PEPD	QSCN6	SRP9	USP5
APRT	CFL2	EEF1A2	GPC1	IFNG	MAPK1	PFN1	RAB11B	SRPX	VARS
ARCN1	CHID1	EEF1B2	GPC5	IGF2R	MAPRE1	PFN2	RAB1A	SRPX2	VASN
ARHGAP1	CHRDL1	EEF1G	GPI	IGFBP2	MAT2A	PGCP	RAB6A	SSB	VAT1
ARHGDIA	CLEC11A	EEF2	GREM1	IGFBP3	MAT2B	PGD	RAC1	ST13	VCL
ARPC1A	CLIC1	EFEMP2	GRHPR	IGFBP4	MCTS1	PGK1	RAN	ST6GAL2	VCP
ARPC1B	CLIC4	EIF2S3	GRN	IGFBP5	MDH1	PGK2	RANBP5	STAT1	VEGFC
ARPC2	CLSTN1	EIF3S9	GSN	IGFBP7	MDH2	PGLS	RARRES2	STC1	VIL2
ARPC3	CLTC	EIF4A1	GSR	IGKC	MFAP4	PGM1	RARS	STC2	VIM
ARPC4	CLTCL1	EIF4A2	GSS	IL13	MGAT5	PGRMC2	RBMX	STIP1	VPS26A
ARTS-1	CLU	EMILIN1	GSTK1	IL15	MIF	PHGDH	RHOA	SULF1	VPS35
ATIC	CMPK	EML2	GSTO1	IL15RA	MMP1	PHPT1	RNASE4	SVEP1	VTN
ATP5B	CNDP2	ENO1	GSTP1	IL1RAP	MMP10	PICALM	RNH1	SYNCRIP	WARS
ATP6AP1	CNN2	ENO2	GTPBP9	IL2	MMP14	PKM2	RNPEP	TAGLN	WDR1
ATP6AP2	CNN3	ENO3	GZMA	IL21R	MMP2	PLAU	RPL10A	TAGLN2	WNT5A
ATP6V1B2	COL12A1	EPPK1	H2AFY	IL3	MRC2	PLEC1	RPL11	TALDO1	WNT5B
ATP6V1G2	COL18A1	EPRS	HADH	IL6	MRLC2	PLEKHC1	RPL12	TARS	XPO1
B2M	COL1A1	ESD	HARS	IL6ST	MSN	PLOD1	RPL14	TCN2	YKT6
B4GALT1	COL1A2	ETF1	HARS2	IL8	MTAP	PLOD2	RPL18	TCP1	YWHAB
BASP1	COL2A1	ETFB	hCG_1641617	ILF2	MTPN	PLOD3	RPL22	TFPI	YWHAE
BAT1	COL3A1	ETHE1	hCG_2023776	ILF3	MVP	PLS1	RPL30	TGFB1	YWHAG
BBS1	COL4A1	EXT1	HEXA	INHBA	MXRA5	PLS3	RPL5	TGFB2	YWHAH
BCAT1	COL4A2	FAH	HEXB	IQGAP1	MXRA8	PLSCR3	RPL7	TGFBI	YWHAQ
BGN	COL5A1	FAHD1	HGF	ISOC1	MYH11	POSTN	RPLP0	THBS	YWHAZ
BLVRA	COL5A2	FAM129B	HIBCH	ITGA2	MYH14	PPCS	RPLP1	THBS1	

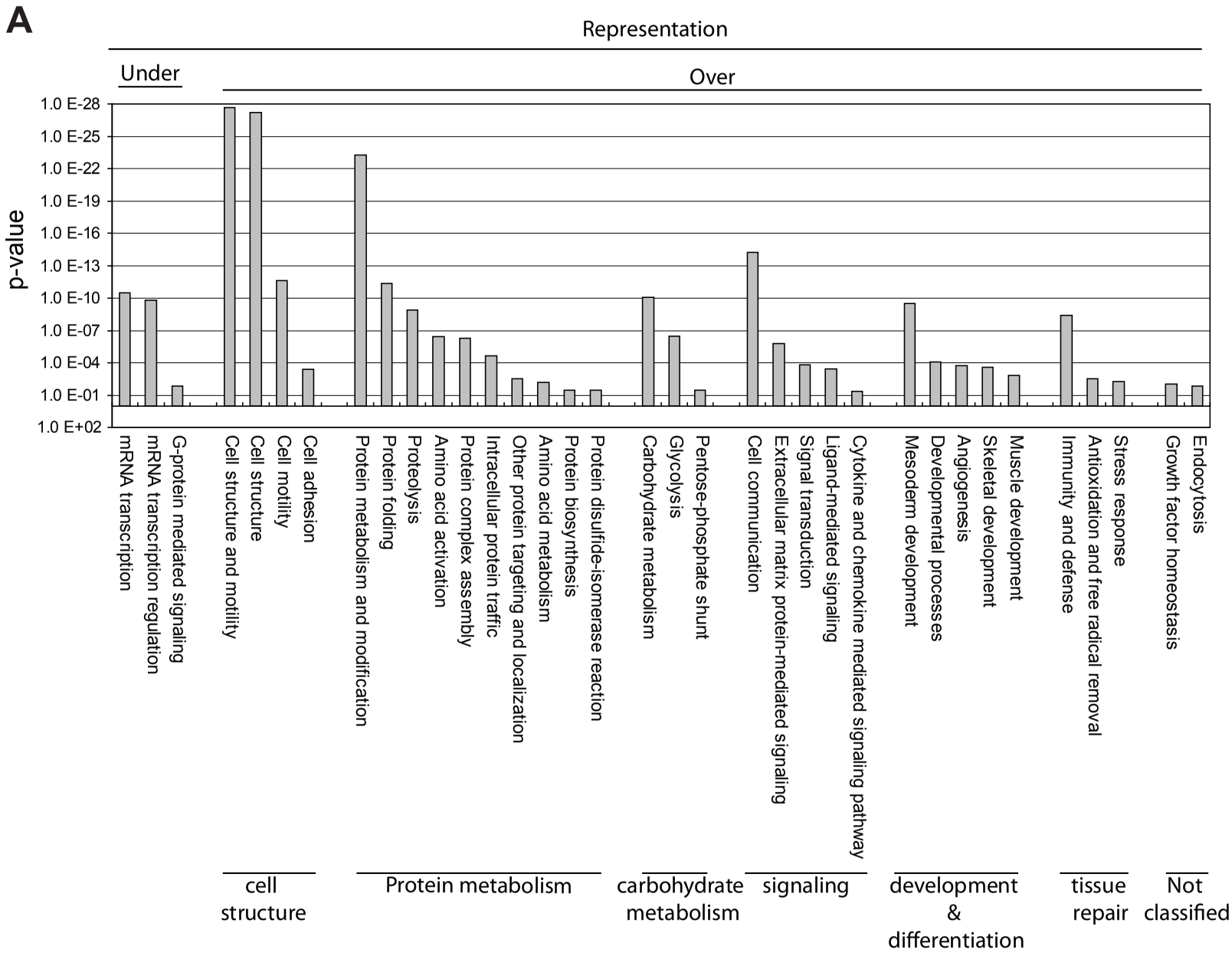
Black font	Identified by LC MS/MS
Grey shade	Identified by antibody array
Underline	Identified by LC MS/MS and antibody array
White font	Identified by LC MS/MS and are present on exosomes secreted by at least 4 different cell types(Olver and Vidal, 2007)

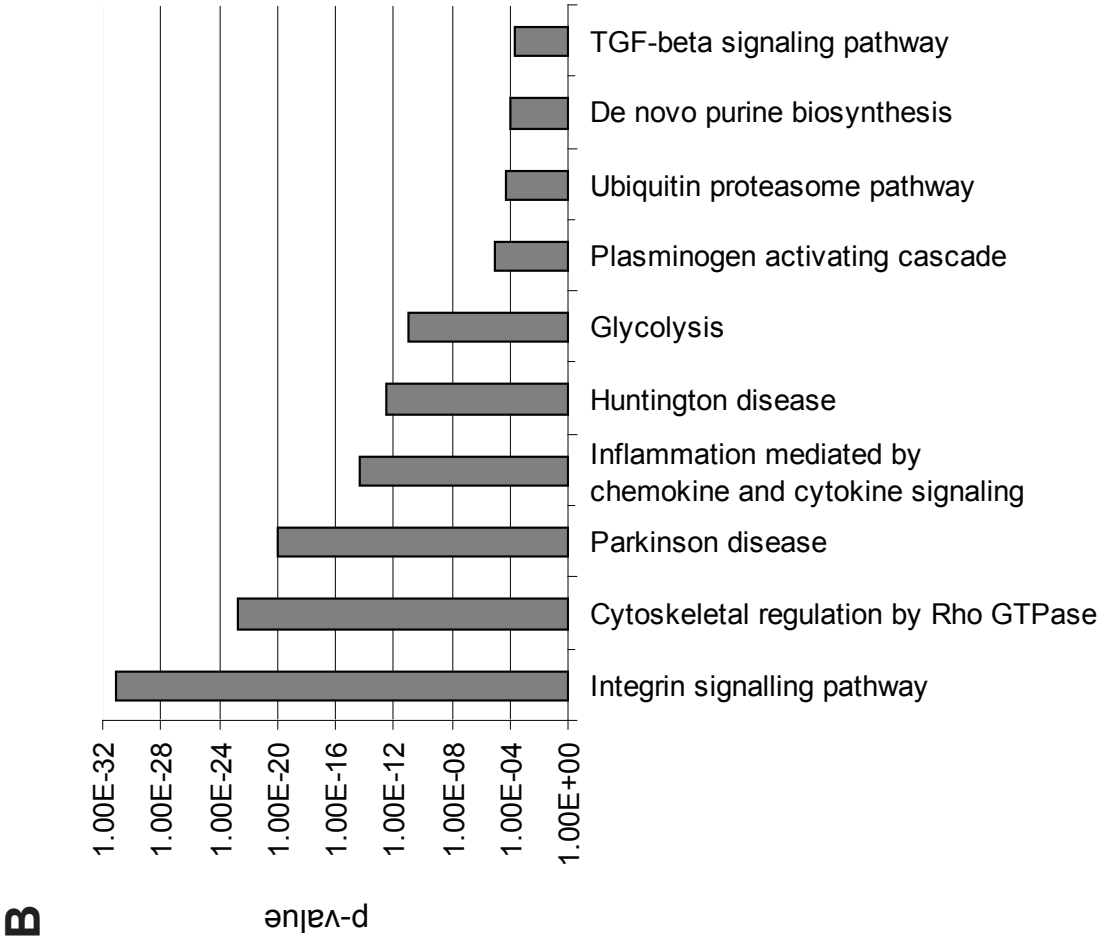
Table 1. Proteomic profile of CM as determined by LC MS/MS and antibody array. For LC MS/MS, 4 independent samples were analyzed, proteins were considered present if detected in at least 3 of 4 samples. For antibody array, 3 independent samples were analyzed, the cytokines and other proteins were considered to be present in the conditioned medium if the signal intensity is 2 fold higher ($p<0.05$) than that in non-conditioned medium.

Supplementary Materials

Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury

Ruenn Chai Lai, Fatih Arslan, Lee May May, Siu Kwan Sze, Andre Choo, Tian Sheng Chen, Manuel Salto-Tellez, Leo Timmers, Chuen Neng Lee, Reida Menshawe El Oakley, Gerard Pasterkamp, Dominique P.V. de Kleijn, Sai Kiang Lim

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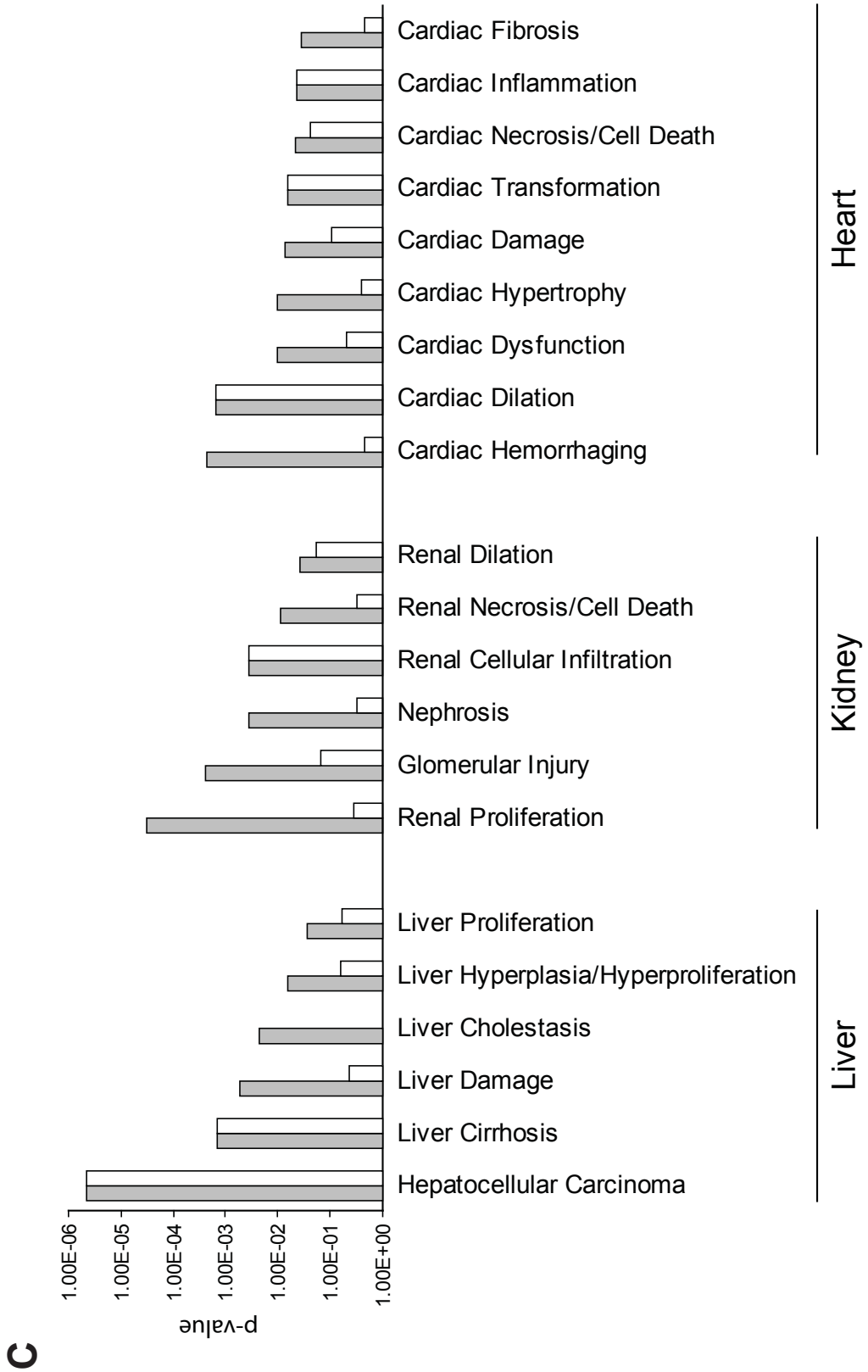
1.00E-04
1.00E+00
Integrin signalling pathway
Cytoskeletal regulation by Rho GTPase
Parkinson disease
Inflammation mediated by chemokine and cytokine signaling
Huntington disease
Glycolysis
Plasminogen activating cascade
Ubiquitin proteasome pathway
De novo purine biosynthesis
TGF-beta signaling pathway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Cardiac Necrosis/Cell Death
Cardiac Transformation
Cardiac Damage
Cardiac Hypertrophy
Cardiac Dysfunction
Cardiac Dilation
Cardiac Hemorrhaging
Renal Dilation
Renal Necrosis/Cell Death
Renal Cellular Infiltration
Nephrosis
Glomerular Injury
Renal Proliferation
Liver Proliferation
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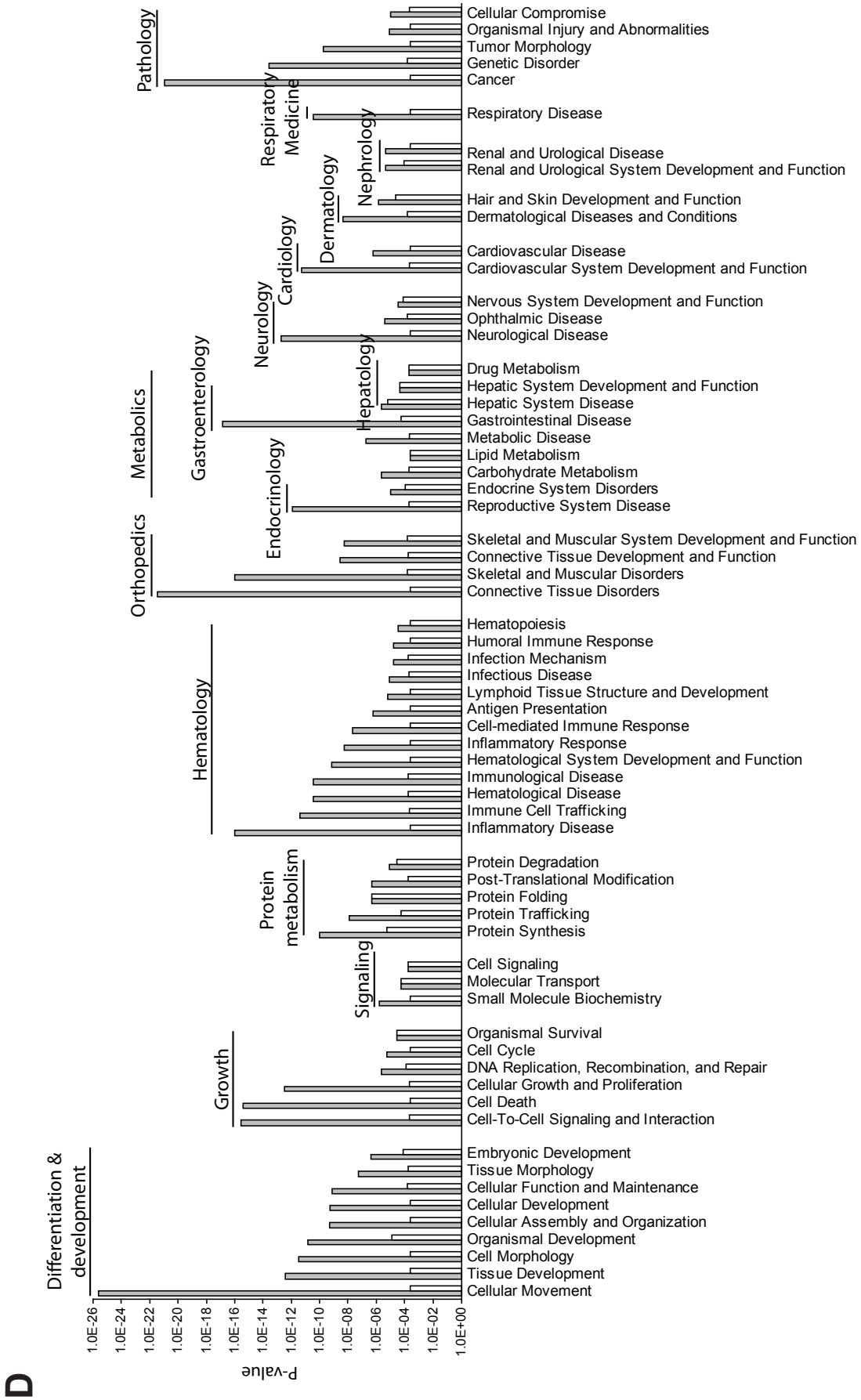
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1.0E-06
1.0E-04
1.0E-02
1.0E+00
Differentiation & development
Cellular Movement
Tissue Development
Cell Morphology
Organismal Development
Cellular Assembly and Organization
Cellular Development
Cellular Function and Maintenance
Tissue Morphology
Embryonic Development
Growth
Cell-To-Cell Signaling and Interaction
Cell Death
Cellular Growth and Proliferation
DNA Replication, Recombination, and Repair
Cell Cycle
Organismal Survival
Signaling
Small Molecule Biochemistry
Molecular Transport
Cell Signaling
Protein metabolism
Protein Synthesis
Protein Trafficking
Protein Folding
Post-Translational Modification
Protein Degradation
Hematology
Inflammatory Disease
Immune Cell Trafficking
Hematological Disease
Immunological Disease
Hematological System Development and Function
Inflammatory Response
Cell-mediated Immune Response
Antigen Presentation
Lymphoid Tissue Structure and Development
Infectious Disease
Infection Mechanism
Humoral Immune Response
Hematopoiesis
Orthopedics
Connective Tissue Disorders
Skeletal and Muscular Disorders
Connective Tissue Development and Function
Skeletal and Muscular System Development and Function
Metabolics
Endocrinology
Reproductive System Disease
Endocrine System Disorders
Carbohydrate Metabolism
Lipid Metabolism
Metabolic Disease
Gastroenterology
Gastrointestinal Disease
Hepatology
Hepatic System Disease
Hepatic System Development and Function
Drug Metabolism
Neurology
Neurological Disease
Ophthalmic Disease
Nervous System Development and Function
Cardiology
Cardiovascular System Development and Function
Cardiovascular Disease
Dermatology
Dermatological Diseases and Conditions
Hair and Skin Development and Function
Nephrology
Renal and Urological System Development and Function
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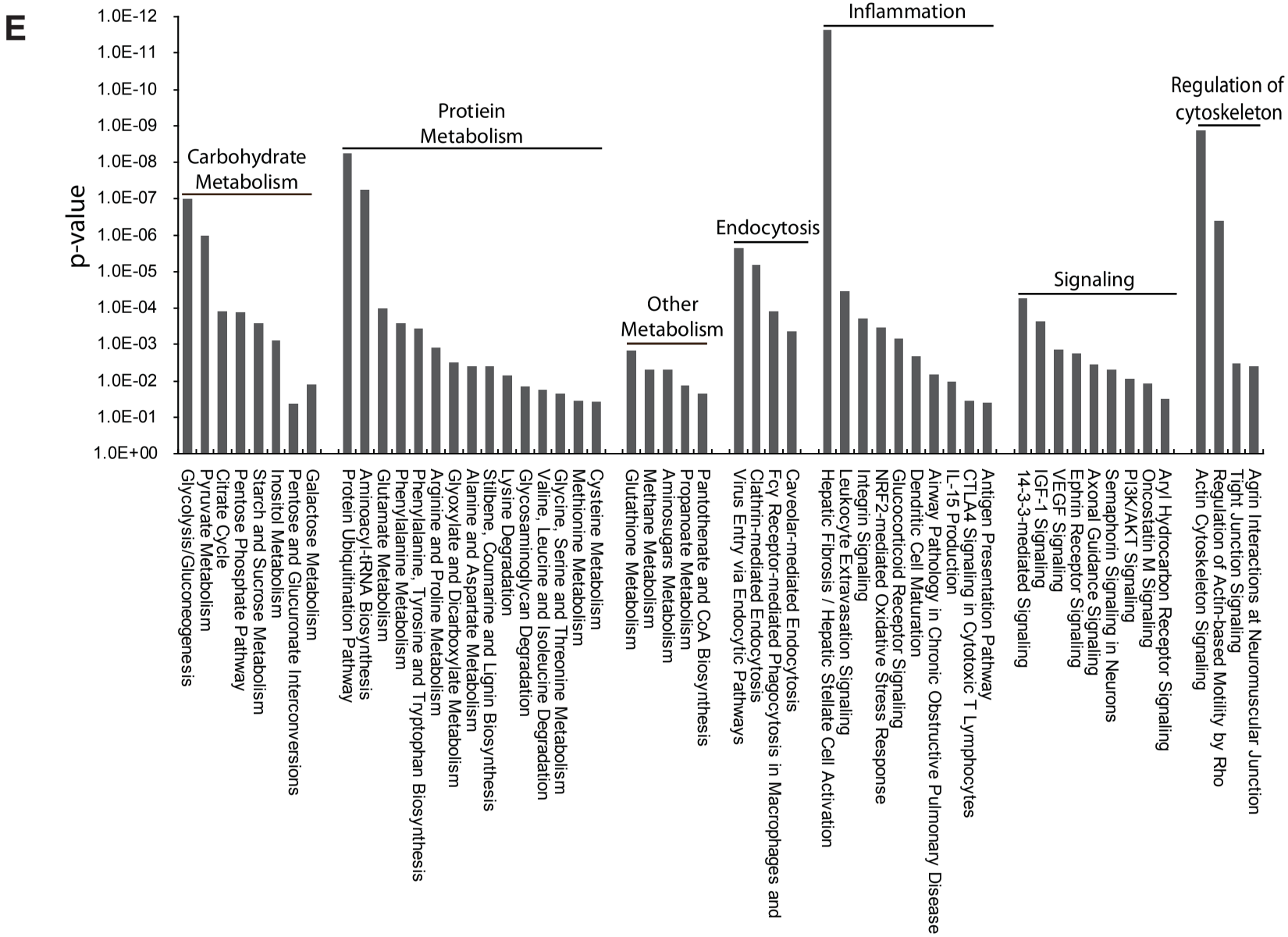
Respiratory Medicine
Respiratory Disease
Pathology
Cancer
Genetic Disorder
Tumor Morphology
Organismal Injury and Abnormalities
Cellular Compromise



Supplementary Figure 1. Analysis of 739 unique gene products of conditioned medium.
Biological processes, biological pathways, toxicology functions, biological functions, were analyzed using PANTHER Expression analysis tools or Ingenuity Pathways Analysis. Biological processes, biological pathways, toxicology functions and biological functions that were significantly over- or underrepresented ($p < .05$) by gene products highly expressed in CM were grouped and graphically presented. A-B) Biological processes and biological pathways analyzed by PANTHER Expression analysis tools for finding statistically significant ontology/pathway associations[1] (PANTHER Pathway version 2.5, http://www.pantherdb.org). C-E) Toxicology functions, biological functions and biological pathways analyzed by Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA). Grey bar and white bar in c and d represent the lowest p-value and highest p-value respectively.

1. Thomas, P.D. et al. Applications for protein sequence-function evolution data: mRNA/protein expression analysis and coding SNP scoring tools. Nucleic Acids Res 34, W645-650 (2006).

Supplementary Materials and Methods

Electron Microscopy

Formvar-coated carbon grids were coated with a drop of 0.01% Poly Lysine for 1 minute before excess fluid was drained off with a filter paper. 50×concentrated samples of either MSC-CM or NCM were pipetted onto the labeled grids and after one minute, excess samples were drained off with a filter paper. A drop of 1% pH 6 phosphotungstic acid was then added onto each grid and after one minute, excess liquid was drained off with a filter paper leaving a thin monolayer that was allowed to dry thoroughly at room temperature. The samples grids were screened using a high-resolution transmission electron microscope, Philips CM120 and micrographs images were taken.

LC MS/MS analysis

Proteins in two ml of dialyzed CM or NCM were reduced, alkylated and tryptic digested as described. 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 the 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 ml/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 modifications 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.

Antibody array assay

500 μl of conditioned or non-conditioned medium 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 conditioned medium if the signal intensity is 2 fold higher ($p<0.05$) than that in non-conditioned medium.

Protein analysis

Analysis of proteins by western blot hybridization was performed using standard protocols. Briefly, proteins were denatured, separated on 4-12% polyacrylamide gels, electroblotted onto a nitrocellulose membrane and probed with antibodies against human CD9, CD81, Alix and SOD-1 (Santa Cruz Biotechnology, Santa Cruz, CA) followed by horseradish peroxidase-coupled secondary antibodies against the mouse primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The blot was then incubated with a chemiluminescent HRP substrate to detect bound primary antibody, and therefore the presence of the antigen.

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 μl. The % area of the exosome peak *vs.* all other peaks was integrated from the UV-visible detector. The hydrodynamic radius, R_h, was computed by the QELS and Dawn 8 detectors. The highest count rate (Hz) at the peak apex was taken as the R_h. Peaks of the separated components visualized at 220 ηm were collected as fractions for further characterization studies.

PAPER TWO

Derivation and Characterization of Human Fetal MSCs: An Alternative Cell Source for Large-Scale Production of Cardioprotective Microparticles

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Journal of Molecular and Cellular Cardiology 48 (2010) 1215–1224

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Derivation and characterization of human fetal MSCs: An alternative cell source for large-scale production of cardioprotective microparticles

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ARTICLE INFO

Article history:
Received 11 September 2009
Received in revised form 15 December 2009
Accepted 28 December 2009
Available online 11 January 2010

Keywords:
Fetal mesenchymal stem cells
Paracrine secretion
Microparticles
Cardioprotection

ABSTRACT

The therapeutic effects of mesenchymal stem cells (MSCs) transplantation are increasingly thought to be mediated by MSC secretion. We have previously demonstrated that human ESC-derived MSCs (hESC-MSCs) produce cardioprotective microparticles in pig model of myocardial ischemia/reperfusion (MI/R) injury. As the safety and availability of clinical grade human ESCs remain a concern, MSCs from fetal tissue sources were evaluated as alternatives. Here we derived five MSC cultures from limb, kidney and liver tissues of three first trimester aborted fetuses and like our previously described hESC-derived MSCs; they were highly expandable and had similar telomerase activities. Each line has the potential to generate at least 10^{16-19} cells or 10^{7-10} doses of cardioprotective secretion for a pig model of MI/R injury. Unlike previously described fetal MSCs, they did not express pluripotency-associated markers such as Oct4, Nanog or Tra1-60. They displayed a typical MSC surface antigen profile and differentiated into adipocytes, osteocytes and chondrocytes *in vitro*. Global gene expression analysis by microarray and qRT-PCR revealed a typical MSC gene expression profile that was highly correlated among the five fetal MSC cultures and with that of hESC-MSCs (r^2>0.90). Like hESC-MSCs, they produced secretion that was cardioprotective in a mouse model of MI/R injury. HPLC analysis of the secretion revealed the presence of a population of microparticles with a hydrodynamic radius of 50–65 nm. This purified population of microparticles was cardioprotective at ~1/10 dosage of the crude secretion.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells that have a limited but robust potential to differentiate into mesenchymal cell types, e.g. adipocytes, chondrocytes and osteocytes, with negligible risk of teratoma formation. MSC transplantation has been used to treat musculoskeletal injuries, improve cardiac function in cardiovascular disease and ameliorate the severity of graft-versus-host-disease [1]. In recent years, MSC transplantations have demonstrated therapeutic efficacy in treating different diseases but the underlying mechanism has been controversial [2–9]. Some reports have suggested that factors secreted by MSCs [10] were responsible for the therapeutic effect on arteriogenesis [11], stem cell crypt in the intestine [12], ischemic injury [9,13–18], and hematopoiesis [19,20].

We have recently demonstrated that human MSCs derived from human embryonic stem cells (hESC-MSCs) [21] secrete >200 proteins [22] and that a single bolus administration of hESC-MSCs conditioned medium (CM) 5 min prior to reperfusion significantly reduced infarct size by 60% and improved cardiac function in a pig and mouse model of myocardial ischemia/reperfusion (MI/R) injury [23]. In addition, this cardioprotection was mediated by large complexes of about 50–100 nm in diameter. The size of these large secreted complexes suggests that

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doi:10.1016/j.yjmcc.2009.12.021

they are microparticles which are broadly defined as secreted membrane particles in the size range of 0.05–1 μm [24].

A requisite for translating cardioprotective MSC secretion into clinical applications is a clinical grade MSC source but this is currently limited by restricted access to clinical grade hESCs. Therefore, alternative tissue or cell sources that are amenable to the generation of highly expandable, clinical grade MSCs have to be developed. Here we examined fetal tissues as a candidate tissue source.

Five MSC cultures, Fllb, F1ki, F2lb, F3lb and F3li were generated from limb (lb), kidney (ki) and liver (li) tissues of three fetuses in three independent experiments. These fetal MSCs fulfilled the defining criteria of a MSC. They were highly proliferative. Each line has the potential of generating 10^{16-19} cells, and therefore the capacity to produce large amount of secretion. More importantly, these cells produce secretion that reduced infarct size in a mouse model of MI/R injury [23]. Like the secretion of hESC-MSCs, this cardioprotection was also mediated by large complexes. Fractionation of the secretion by size exclusion on an HPLC revealed the presence of a population of homogenously sized particles with a hydrodynamic radius of 50–65 nm and these particles were cardioprotective in a mouse model of MI/R injury. The size of these large secreted complexes suggests that they are microparticles which are broadly defined as secreted membrane particles in the size range of 0.05–1 μm [24].

2. Materials and methods

2.1. Derivation of fetal tissue derived MSCs

The collection of fetal tissue was carried out under a KK Women's and Children's Hospital (KKH) IRB approved protocol (EC200804062) in accordance with guidelines from Singapore Bioethics Advisory Committee [25] which stated that the decision to donate the fetal tissue must be made independently from any decision to abort. Only patients who have already consented to Termination of Pregnancy (TOP) in KKH Outpatient Clinic were recruited. Recruitment was carried out in strict adherence to KKH IRB's regulations to ensure patient's rights and privacy, and to provide confidential counseling for patient's fully informed consent to voluntary donation. TOP for fetal abnormalities and sexual assault cases and in minor (16 and below) were excluded. Patients with medical problems were also excluded.

The aborted specimens were collected in special sterile plastic bottles and sent to the hospital's Department of Laboratory Medicine for full pathological examinations. Appropriate pieces of fetal tissues were dissected, washed several times in sterile saline, minced, and placed in DMEM supplemented with 10% serum replacement medium, EGF (20 ng/ml) and FGF2 (20 ng/ml) to attach to plastic tissue culture dishes for 24–48 h. Under this condition, MSCs whose defining characteristic is adherence to plastic migrated out of the tissues and adherence to the plastic culture dish. The large tissue pieces are then washed off leaving a homogenous cell culture. The cells were maintained at 25%–80% confluency or 15–50,000 cells per cm^2 and were split 1:4 at confluency by trypsinization. On reaching 2×10^7 cells usually within 2 weeks, the culture was designated P1. Master cell banks of early passage cells grown in culture medium supplemented with animal replacement medium were set up for all the lines. For all experimental work described in this study, cells at p10 were expanded in HuES Expansion medium containing 10% ES cell fetal bovine serum 1% L-glutamine, 1% non essential amino acids, and 88% DMEM (high glucose, no sodium pyruvate). All medium components were obtained from Invitrogen Corporation, Carlsbad, CA. Differentiation of the fetal MSCs to adipocytes, chondrocytes and osteocytes was performed using adipogenic, chondrogenic and osteogenic hMSC Differentiation BulletKits, respectively (Lonza, Walkersville, MD) as per manufacturer' instructions. Karyotyping by G-banding was performed at the Cytogenetics Laboratory, KKH.

2.2. Telomerase activity

Relative telomerase activity was measured by SYBR® Green real time quantitative telomeric repeat amplification protocol assay using a modified method as described by Wege et al. [26]. Briefly, 3 million cells were harvested and cell lysate was prepared using a commercially available mammalian cell extraction kit (BioVision, Singapore). The composition of the reagents for the PCR amplification was 1 μg of protein cell lysate, 10 μl of 2× SYBR Green Super Mix (BioRad, Singapore) with 0.1 μg of TS primer (5′-AATCCGTCGAGCAGACTT-3′), 0.1 μg of ACX primer (5′-GCGCGG[CTTACC]^{3}CTAACC-3′) and 10 mM EGTA in a total volume of 25 μl. The reaction was first incubated at 25 °C for 20 min to allow the telomerase in the cell lysate to elongate the TS primers followed by 2 min incubation at 95 °C to inactivate telomerase activity and denature the primers. The telomerase product was amplified by PCR for 40 cycles of 95 °C for 30 s and 60 °C for 90 s. The relative telomerase activity was assessed against that of HEK cells using the threshold cycle number (or Ct value) for 1 μg protein cell lysate.

2.3. Surface antigen analysis

Expression of cell surface antigens on fetal MSCs was analyzed using flow cytometry. The cells were trypsinized for 5 min, centrifuged, resuspended in culture media and incubated in a bacterial culture dish for 1 h in a 37 °C, 5% CO_2 incubator. The cells were then collected, centrifuged, and washed in 2% FBS. A total of 2.5×10^5 cells were then incubated with each of the following conjugated monoclonal antibodies: CD29-PE, CD44-FITC, CD49a-PE, CD49e-PE, CD105-FITC, CD166-PE, CD73-FITC, CD34-FITC, and CD45-FITC (PharMingen, San Diego, CA) for 60 min on ice. After incubation, cells were washed and resuspended in 2% FBS. Nonspecific fluorescence was determined by incubation of similar cell aliquots with isotype-matched mouse monoclonal antibodies (PharMingen, San Diego, CA). Data were analyzed by collecting 20,000 events on a BD FACSCalibur™ Flow Cytometer (BD Biosciences, San Jose, CA) instrument using CELLQuest software.

2.4. Quantitative RT-PCR

Total RNA was extracted from cells using TRIzol® LS Reagent (Invitrogen Corporation, Carlsbad, CA) according to manufacturer's instruction. Total RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA) that was based on oligo-dT primed reverse transcription. Real time PCR was performed on a StepOne™ Plus Real-Time PCR System (Applied Biosystems Inc, Foster City, CA) using 2× Fast SYBR® Green Master Mix (Applied Biosystems Inc, Foster City, CA) according to manufacturer's instruction. The primers for OCT4 were 5′-AGTGAACAGGGAATGGGTGAA-3′ and 5′-AAG CGG CAG ATG GTC GT-3′, and for SOX2 were 5′-TGAGAGAAAGAAGAGGA-GAGA-3′ and 5′-TGGGGGAAAAAAAGAGAGAGG-3′.

2.5. Illumina gene chip analysis

Total RNA was prepared in technical triplicates from different passages of F1lb (p10, p12, p14), F1ki (p12, p14, p16), F2lb (p10, p12, p14), F3lb (p10, p12, p14) and F3li (p10, p16), and from two technical replicates of the previously described hESC-MSCs line, Hues9.E1 (p19). Five hundred nanograms of RNA was converted to biotinylated cRNA using the Illumina RNA Amplification Kit (Ambion, Inc., Austin, TX) according to the manufacturer's directions. Seven hundred fifty nanograms of the biotinylated cRNA was hybridized to the Sentrix HumanRef-8 Expression BeadChip Version 3 (Illumina, Inc., San Diego, CA), and washing and scanning were performed according to the Illumina BeadStation 500× manual. The data were analyzed using Genespring GX 10. Quantile normalization was performed by a shift to

75th percentile, and the normalized data were baseline transformed to the median of all samples.

2.6. SDS–PAGE analysis and western blot hybridization

For SDS–PAGE analysis, total proteins in CM were separated on 4–12% SDS–polyacrylamide gels and stained with silver. For western blot hybridization, the proteins were electroblotted onto a nitrocellulose membrane after first separating on an SDS–PAGE. The membrane was blocked and incubated with the primary anti-human antibodies that included 1:60 dilution of mouse anti-CD9, 1:60 dilution of mouse anti-CD81, 1:56 dilution of mouse anti-Alix, 1:200 dilution of mouse anti-pyruvate kinase (PK), 1:60 dilution of mouse anti-SOD-1 or 1:60 dilution of goat anti-TSP-1. The blot was then incubated with a horseradish peroxidase-coupled secondary antibody. The secondary antibodies used were 1:1250 or 1:1364 dilution of goat anti-mouse IgG or 1:1364 dilution of donkey anti-goat IgG. All antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA except mouse anti-PK which is 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 an X-ray film.

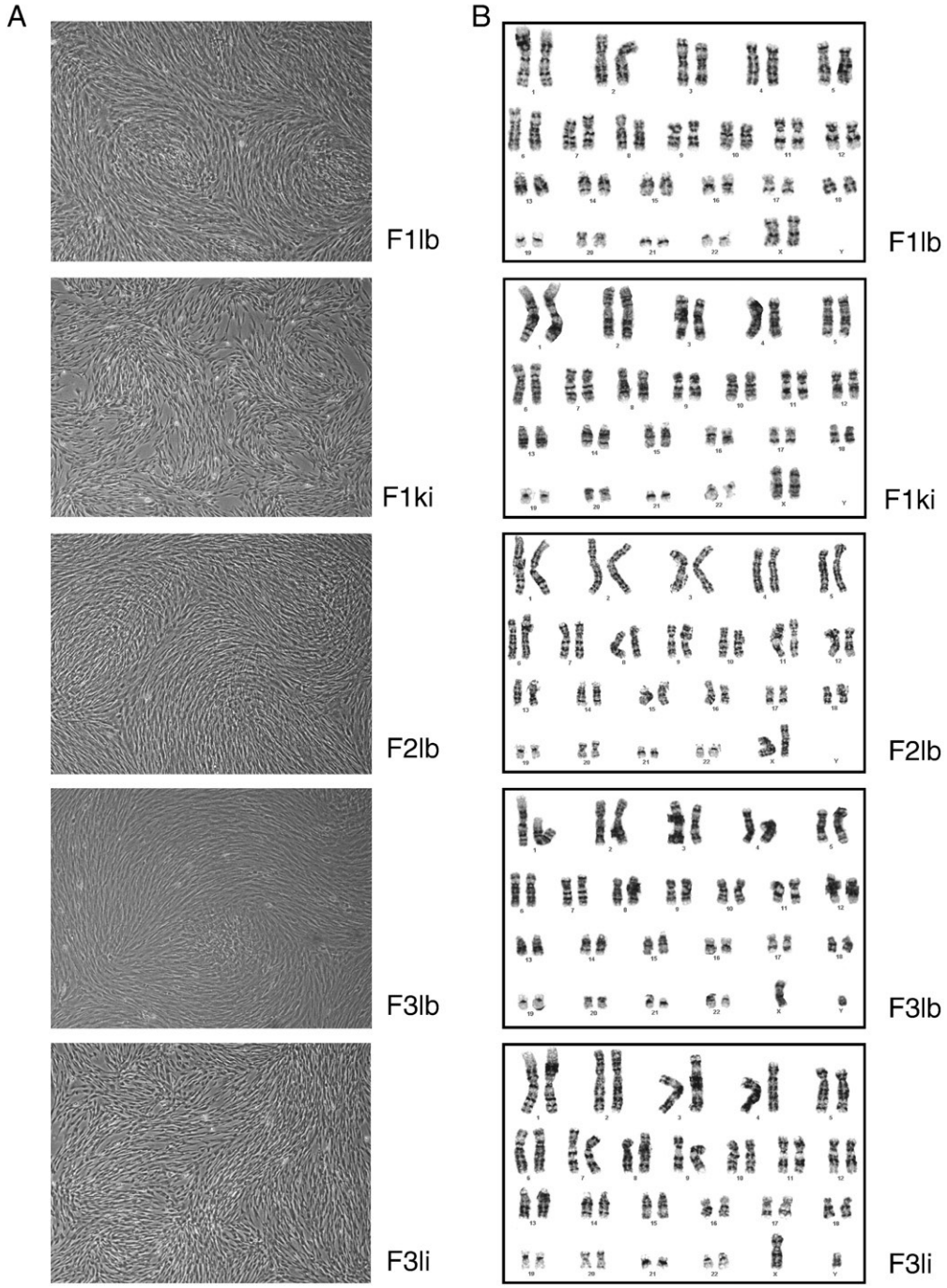

Fig. 1. Characterization of fetal MSC cultures. (A) Cellular morphology under phase contrast. Representative images of the five different MSCs, F1lb (p8) and F1ki (p8) derived from the limb and kidney tissues of the same fetus; F2lb (p8) derived from the limb of a second fetus; and F3lb (p8) and F3li (p8) derived from the limb and liver tissues of the same third fetus. (B) Karyotype analysis by G-banding was performed each of the fetal MSC cultures, F1lb (p10) F2lb (p10), F3lb (p10), F1ki (p12), and F1li (p12).

2.7. HPLC purification of microparticles

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, 6×40 mm and TSK gel G4000 SWXL, 7.8×300 mm from Tosoh Corporation (Tokyo, Japan). The detectors Dawn 8 (light scattering), Optilab (refractive index) and QELS (dynamic light scattering) were connected in a 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, that is, 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 min 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 nm and the column was oven equilibrated to 25°C. The elution mode was isocratic and the run time was 40 min. The volume of sample injected ranged from 50 to 100 μl. 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 nm were collected as fractions for further characterization studies.

2.8. Testing secretion for cardioprotection

The secretion was prepared by growing the fetal MSCs in a chemically defined serum-free culture medium for 3 days as previously described [22]. Briefly, cells at p12 were first expanded in serum-containing culture medium as described above. At p15, 80% confluent cell cultures were washed three times with PBS and then incubated in a chemically defined medium consisting of DMEM without phenol red (Invitrogen Corporation, Carlsbad, CA) and supplemented with insulin, transferrin, and selenoprotein (ITS) (Invitrogen Corporation, Carlsbad, CA), 5 ng/ml FGF2 (Invitrogen Corporation, Carlsbad, CA), 5 ng/ml PDGF AB (Peprotech, Rocky Hill, NJ), glutamine–penicillin–streptomycin, and β-mercaptoethanol overnight. The cell culture was then washed with PBS and replaced with fresh chemically defined medium for another 3 days to produce the conditioned medium. This CM was collected and clarified by centrifugation at 500× *g*. The clarified CM concentrated 50 times by reducing its volume by a factor of 50 using a tangential flow filtration system with membrane MW cutoff of 100 kDa (Satorius, Goettingen, Germany). The use of a membrane MW cutoff of 100 kDa allows molecules with MW of less than 100 kDa to pass through the filter resulting a preferential loss of molecules less than 100 kDa. The concentrated CM was then sterilized by filtration through a 220 nm filter.

The CM was tested in a mouse model of MI/R injury. MI was induced by 30 min left coronary artery (LCA) occlusion and subsequent reperfusion. Five minutes before reperfusion, mice were intravenously infused with 200 μl saline diluted CM containing 3 μg protein for Hues9.E1 (hESC-MSCs) CM or 150 μg protein for fetal MSC CM or 10 μg protein for HPLC F1 via the tail vein. Control animals were infused with 200 μl saline. After 24 h of reperfusion, infarct size (IS) as a percentage of the area at risk (AAR) was assessed using Evans' blue dye injection and TTC staining as described previously [23].

2.9. Statistical analysis

Two-way ANOVA with post-hoc Dunnett was used to test the difference in infarct size between groups. Correlation coefficient of each pairs of array was assessed using Pearson correlation test.

3. Results

3.1. Generating MSC cultures from human fetal tissues

We generated five MSC cultures from fetal limb (F1lb, F2lb, F3lb), kidney (F1ki) and liver (F3li) tissues of three fetuses in three independent experiments using feeder- and serum-free culture condition as previously described [21]. A homogenous culture of putative fibroblast-like MSCs migrated out of the tissues and adhered to the plastic culture dish, 2 days after fetal tissues were plated on gelatinized tissue culture plates. This observation was consistent with the defining characteristic of MSCs i.e. adherence to plastic. The large tissue pieces were then washed off leaving a homogenous cell culture. This procedure was performed on five different fetal tissues originating from 3 fetuses in 3 independent experiments. Each time, a homogenous culture of putative MSCs was obtained that form a typical fingerprint whorl at confluency (Fig. 1A). The cultures were designated P1 when 2×10^7 cells were generated. The average population doubling time of all five cultures was between 48 and 72 h and was most optimal at between 25% and 80% confluency or 15–50,000 cells per cm^2. The cells could be maintained in continuous culture for at least 20 passages at a 1:4 split with minimal changes in population doubling time. The karyotype of all five cultures at p10–12 was normal i.e. 46 XX or 46XY as determined by G-banding (Fig. 1B). At passages 14, 16 and 18, telomerase activity in all five MSC cultures was determined and the average cellular telomerase activity over three passages in each of the five cultures was equivalent to that of the previously described Hues9.E1 hESC-MSCs [21] (Fig. 2). All five cultures could be expanded to at least passage 20 without obvious changes in the population doubling time. At a 1:4 split ratio and starting with 10^7 cells, each line would generate 10^{16} cells at passage 15 or 10^{19} cells at passage 20.

3.2. Assessment of fetal cultures as MSCs

The five putative MSC cultures derived from fetal tissues were assessed according to the ISCT minimal criteria for the definition of human MSCs [27]. All five presumptive MSC cultures derived from three different fetuses were grown on plastic culture dishes as a monolayer of adherent spindle-shaped cells (Fig. 1A). They were all $CD29^+$, $CD44^+$, $CD49a^+$ $CD49e^+$, $CD105^+$, $CD166^+$, MHC I^+, $CD34^-$

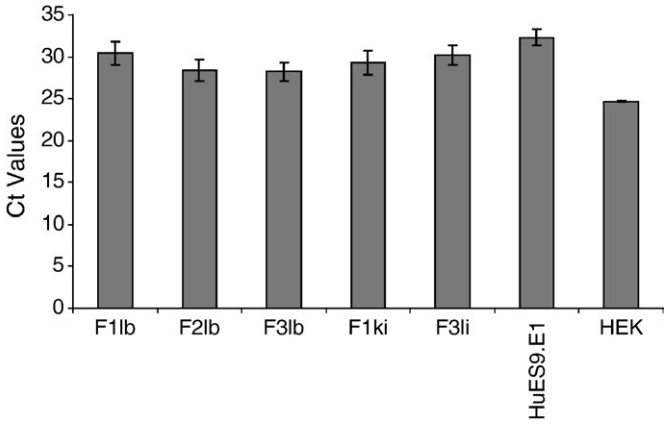

Fig. 2. Telomerase activity in hESC-MSCs and fetal MSCs. Relative telomerase activity was measured by real time quantitative telomeric repeat amplification protocol. This qPCR-based assay quantifies product generated *in vitro* by telomerase activity present in the samples. The relative telomerase activity which is directly proportional to the amount of telomerase products was assessed by the threshold cycle number (or Ct value) for 1 μg protein cell lysate. Hues9.E1 referred to a previously described hESC-MSCs line and HEK is a human embryonic kidney cell line. The Ct value for each fetal MSCs was the mean for three passages, P16, P18, and P20, and that for Hues9.E1 was the mean for two passages, P20 and P22. The assay was performed in triplicate for each passage.

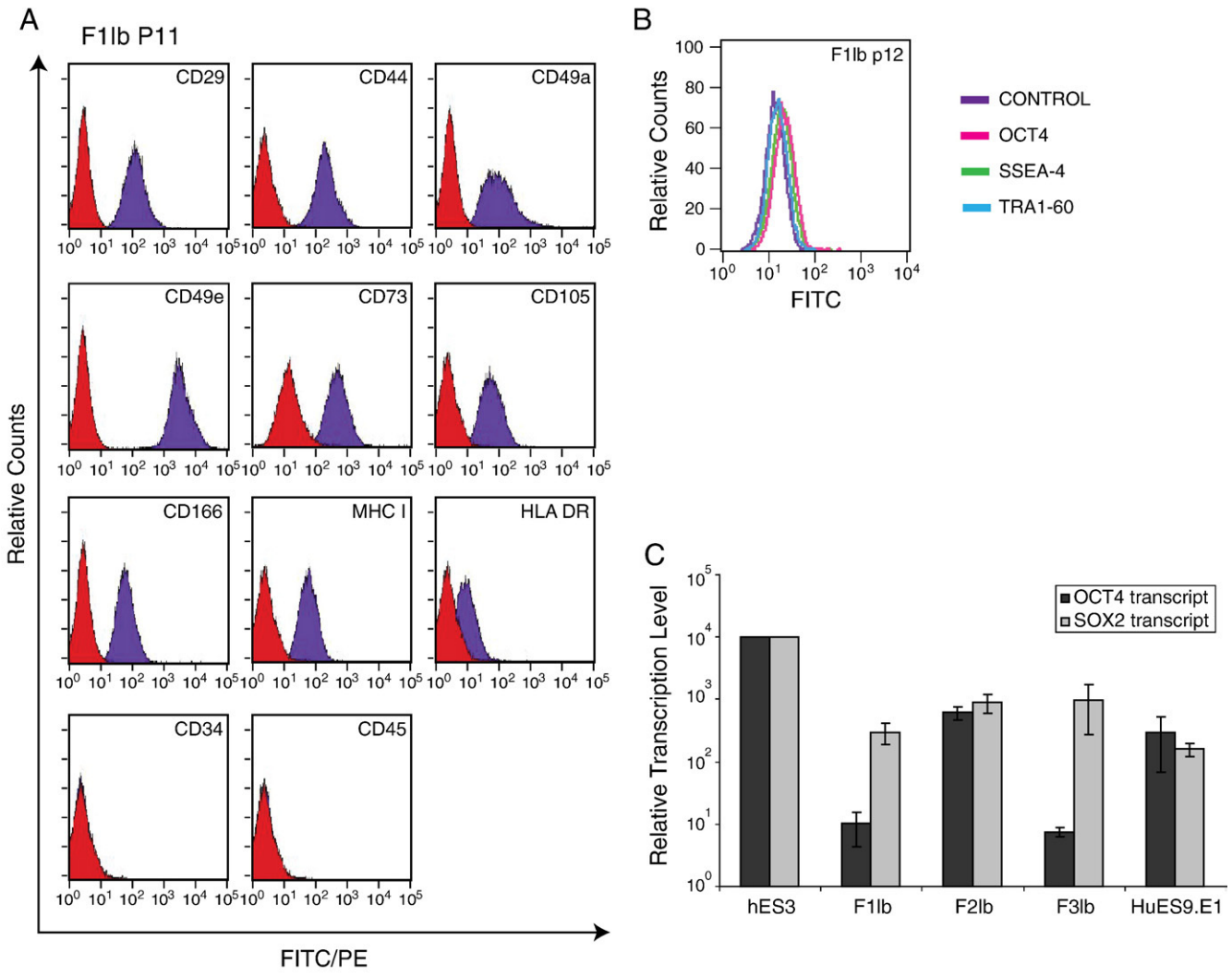

Fig. 3. Marker profiling. (A, B) F1lb MSCs at p11 or p12 were stained with a specific antibody conjugated to a fluorescent dye and analyzed by FACS. Nonspecific fluorescence was determined by incubation of similar cell aliquots with isotype-matched mouse monoclonal antibodies. (C) Relative transcription level of OCT4 and SOX2 was measured using quantitative RT-PCR. hES3, a human embryonic stem cells line was set as the baseline for comparison.

and $CD45^{-}$ as represented by F1lb MSCs (Fig. 3A). We observed that F1lb MSCs were $HLA\text{-}DR^{lo}$ but the remaining four cultures were $HLA\text{-}DR^{-}$. In contrast to previous reports [28–37], these fetal MSCs-like hESC-MSCs did not express pluripotency-associated proteins Oct4, SSEA-4, and Tra1-60 as exemplified by F1lb MSCs (Fig. 3B). However, transcripts of OCT4 and SOX2 were readily detected by real time PCR but their levels were at least ten times lower than those in hES3 human ESCs (Fig. 3C). All five presumptive fetal MSC cultures could be induced to differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* (Fig. 4).

3.3. Gene expression profile

Genome-wide gene expression profiling of the fetal MSCs and hESC-MSCs was performed using microarray hybridization to assess 1) the relatedness among the five fetal MSC cultures derived from three different tissues and 2) the relatedness between the fetal MSC cultures and hESC-MSCs. Microarray hybridization was performed in duplicate on Sentrix Human Ref-8 Expression BeadChip version 3 (Illumina, Inc., San Diego, CA) using RNA from two or three different passages of the MSC cultures. The gene expression profile between

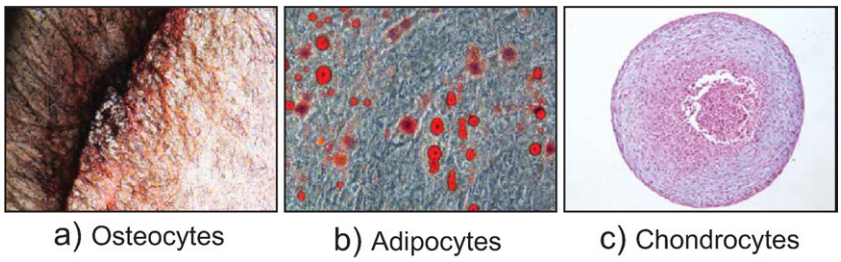

Fig. 4. Differentiation of fetal MSCs. Fetal MSCs were induced to undergo osteogenesis, adipogenesis and chondrogenesis. After a) osteogenesis, b) adipogenesis and c) chondrogenesis, the differentiated cells were stained with von Kossa stain, Oil Red and Alcian blue, respectively. Images of differentiated fetal MSCs as represented by differentiated F3lb MSCs at 100×magnification.

different passages of each culture, between the five cultures or between each fetal MSC culture and hESC-MSCs was highly similar with a correlation value of >0.9 (Fig. 5).

3.4. Cardioprotective activity of secretion

Secretion by two of the fetal MSC cultures, F1lb and F1ki were tested for cardioprotective activity in a mouse model of myocardial ischemia and reperfusion injury as previously described [23]. Briefly, the cultures were grown in a chemically defined medium and the secretion harvested as we have previously described [22]. A typical culture of 10^9 cells yielded 100 mg protein. The gross protein composition of secretion from both F1lb and F1ki as determined by silver staining of proteins resolved on a one-D SDS–PAGE appeared similar to each other, to that of Hues9.E1 hESC-MSCs and also to that from F3lb derived from fetal limb tissues of a different sex (Fig. 6A). However, the relative abundance of specific proteins such as TSP-1, SOD-1, CD81 and CD9 was different among all the secretions (Fig. 6B). Nevertheless, the secretion from either F1lb or F1ki when administered to a mouse model of MI/R injury significantly reduced infarct size to the same extent as mice treated with hESC-MSC CM (Fig. 6C–G). Conditioned medium from F1lb, F1ki and hESC-MSCs reduced the relative infarct size (IS) by 50%, 42% and 39% respectively ($p<0.05$; Fig. 6G). The area at risk (AAR) as a percentage of the left ventricular (LV) wall was similar in all the mice tested (Fig. 6G).

3.5. Microparticles mediated the cardioprotection effects of the secretion

We had previously shown that the cardioprotection effects of secretion from hESC-MSCs were mediated by large complexes of ~1000 kDa [23]. To determine if there were such complexes in the secretion of the fetal MSCs, the CM was fractionated by size exclusion on an HPLC column. We observed five fractions that were present in the CM but not in the non-conditioned medium (NCM) (Fig. 7A). NCM was essentially culture medium that had not been exposed to cells but was processed, concentrated and filtered in the same manner as CM. It was previously shown to have no cardioprotective effect and was equivalent to saline in this respect [23]. These five fractions, F1–F5 therefore represented secretion from the MSCs. In a size exclusion fractionation where larger molecules are eluted faster than smaller ones, we observed that only proteins in fraction F2 to F5 followed this principle of fractionation. Proteins in the fastest eluting F1 fraction contained proteins that spanned in entire MW spectrum of F2 to F5. This suggested that the proteins in F1 were in large aggregates (Fig. 7B). To confirm this, sizes of molecules in the five fractions were determined by dynamic light scattering analysis which has a detection range of 1 to 500 nm according to the manufacturer specification (Wyatt Technology Corporation, www.wyatt.com). The sizes of molecules in fractions F2 to F5 were too heterogeneous to be determined by dynamic light scattering analysis. In spite of the wide MW spectrum of proteins in F1, the size of molecules in F1 fraction was sufficiently homogenous to be determined as having a hydrodynamic radius of 50–65 nm by dynamic light scattering analysis. When administered to the mouse model of MI/R injury as described above, these HPLC-purified microparticles reduced infarct size at <1/10 dosage of the secretion (Fig. 7C–F).

4. Discussion

The trophic effects of MSCs transplantation on ameliorating the deleterious consequences of myocardial ischemia have been implicated in several studies [10]. Transplantation of MSCs into ischemic myocardium has been shown to induce several tissue responses such as an increased production of angiogenic factors and decreased apoptosis [38] that were better explained by secretion of paracrine factors than by differentiation of MSCs, the so-called paracrine hypothesis.

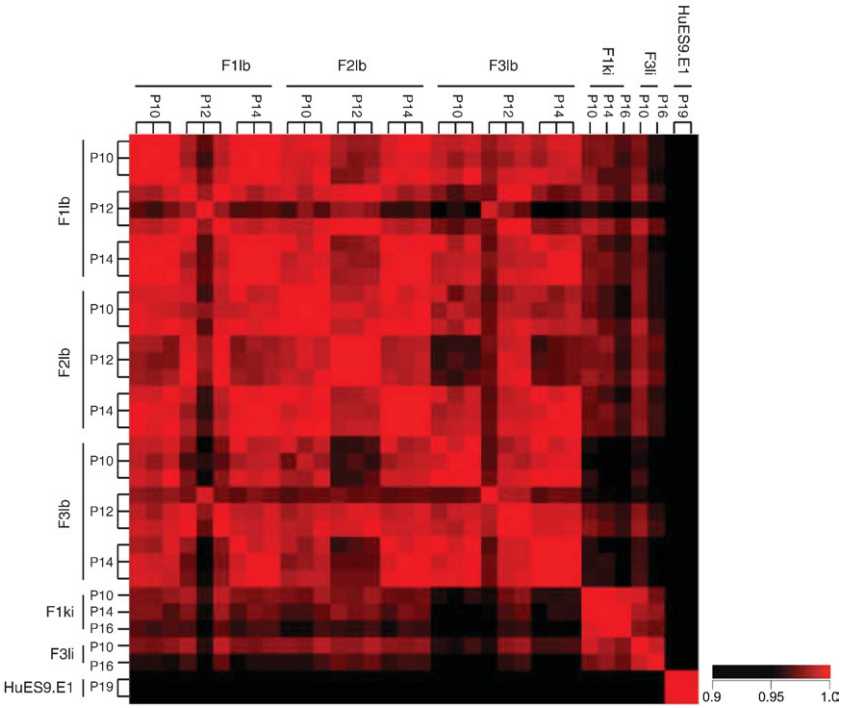

Fig. 5. Gene expression analysis. Total RNA was prepared in technical replicates from different passages of F1lb (p10, p12, p14), F1ki (p10, p14, p16), F2lb (p10, p12, p14), F3lb (p10, p12, p14) and F3li (p10, p16), and from two technical replicates of the previously described hESC-MSCs line, Hues9.E1 (p19). Seven hundred fifty nanograms of biotinylated cRNA from each sample was used for microarray analysis on the Sentrix HumanRef-8 Expression BeadChip Version 3 (Illumina, Inc., San Diego, CA). The gene expression profile of all samples was normalized by a shift to the 75th percentile, baseline transformed to median of all samples, and a heat map of correlation between pairs of array plotted.

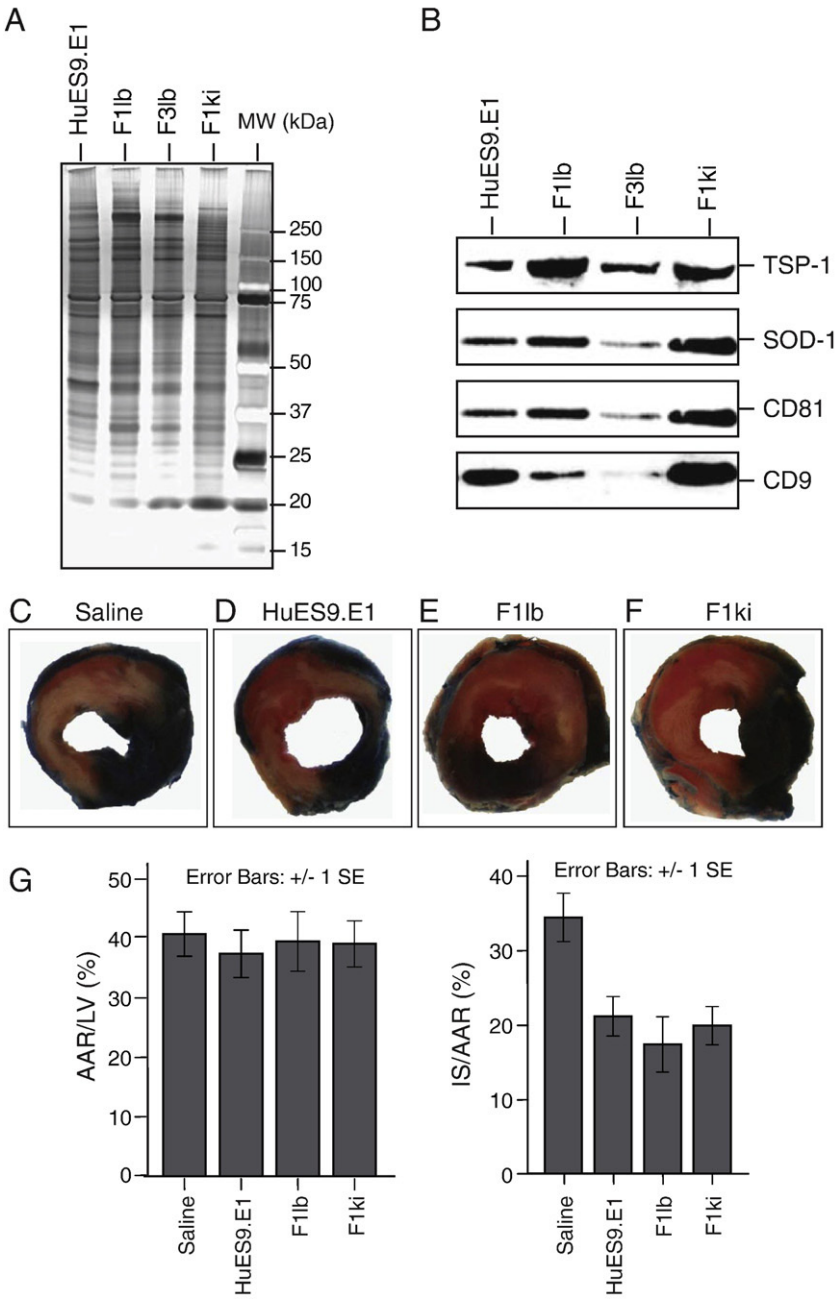

Fig. 6. Cardioprotective secretion. (A) Proteins in culture medium conditioned by hESC-MSCs (Hues9.E1), F1lb, F1ki or F3lb were separated on a 4%–12% SDS–PAGE gradient gel and stained with silver. Two micrograms of proteins was loaded in each lane. (B) Western blot analysis. CM from hESC-MSCs (Hues9.E1), F1lb, F1ki and F3lb at 4, 8, 16 and 16 μg, respectively, were resolved on an SDS–PAGE, electroblotted onto nitrocellulose membrane and probed with antibodies against TSP-1, SOD-1, CD81 and CD9. (C–F) Representative pictures of Evan blue (blue) and TTC (pink) staining on hearts of mice treated with (C) saline, (D) HuES9.E1, (E) F1lb, or (F) F1ki. (G) AAR as a percentage of the left ventricle (LV), showing the amount of endangered myocardium after MI/R injury. All animals were affected to the same extent by the operative procedure, resulting in 39.4 ± 2.0% of AAR among the groups. Infarct size (IS) as a percentage of the area at risk (AAR) upon treatment with saline ($n = 10$), CM from hESC-MSCs ($n = 10$), F1lb-MSCs ($n = 6$) and F1ki-MSCs ($n = 6$). Saline treatment resulted in 34.5 ± 3.3% infarction, whereas conditioned medium from hESC-MSCs, F1lb-MSCs and F1ki-MSCs resulted in 21.2 ± 3.3%, 17.4 ± 3.7% and 19.9 ± 2.6%, respectively. Each bar represents mean ± SEM.

We have recently demonstrated that hESC-MSC secretion reduces infarct size in mouse and pig MI/R injury model [23]. To translate this finding into a clinical application, an important requisite would be a safe, relatively accessible and highly proliferative source of MSCs that has the potential to generate large number of cells to minimize batch to batch variation. Although hESC-MSCs are highly proliferative and have the capacity to generate large number of cells, the number of clinical grade hESC lines and their accessibility is limited. To circumvent this limitation, we derived human MSCs directly from fetal human tissues. The traditionally used bone marrow for deriving MSCs was not an attractive alternative as a single bone marrow aspirate usually generates only ~10^9 cells. We rationalized that fetal tissues being developmentally less mature may generate MSCs with expansion potential equivalent to that of hESC-MSCs.

The fetal MSCs-like hESC-MSCs, fulfilled the basic criteria for MSCs as defined by The International Society for Cellular Therapy [27]. They adhered to plastic, have a typical MSCs-like surface antigen profile as defined by the presence of surface antigens such as CD29, CD44, CD49a, CD49e, CD105, CD166, and MHC I and the absence of surface antigens such as HLA-DR, CD34 and CD45 [39–41], and a typical MSCs differentiation potential that includes adipogenesis, chondrogenesis and osteogenesis. Irrespective of their tissue of origin, the five fetal

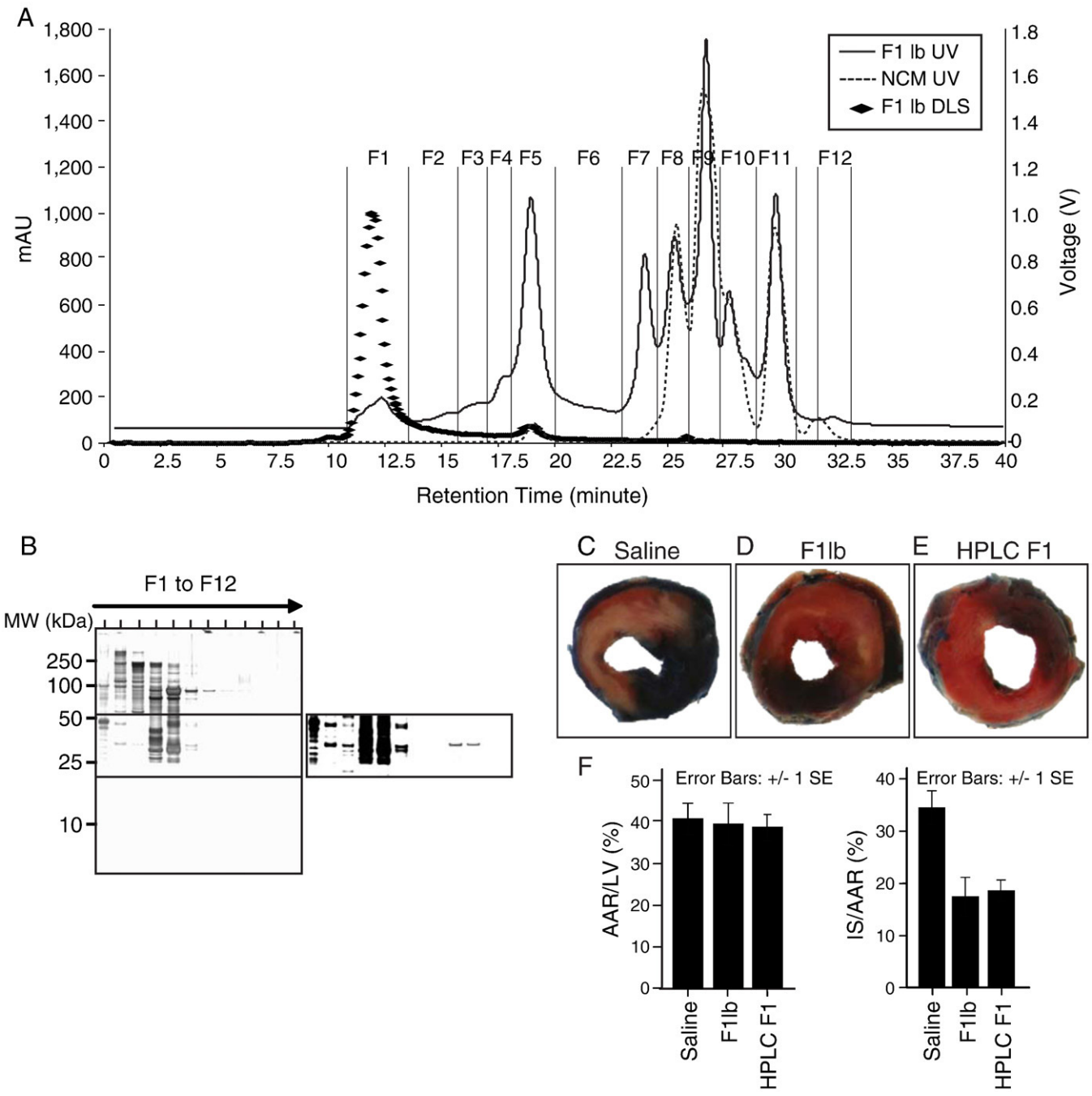

Fig. 7. Cardioprotective HPLC-isolated microparticles. (A) HPLC fractionation and dynamic light scattering of F1lb CM and NCM. F1lb CM and NCM were fractionated on an HPLC using BioSep S4000, 7.8 mm × 30 cm column. The components in F1lb CM or NCM were eluted with 150 mM of NaCl in 20 mM phosphate buffer, pH 7.2. The elution mode was isocratic and the run time was 40 min. The eluent was monitored with a UV–visible detector set at 220 nm and light scattering signal was collected. The solid rhombus represented light scattering signal as measured in voltage; (B) The eluted fractions, F1 to F12 were collected, their volumes were adjusted to 10% of the input volume of CM and equal volume of F1–F12 was separated by gel electrophoresis and stained with silver. (C–E) Representative pictures of Evan blue (blue) and TTC (pink) staining on hearts of mice treated with (C) saline, (D) F1lb, or (E) HPLC F1. (F) Infarct size (IS) as a percentage of the area at risk (AAR) upon treatment with saline ($n = 10$), F1lb CM ($n = 6$) and HPLC F1 ($n = 6$). Saline-treated mice had a 34.5 ± 3.3% relative infarct size while F1lb CM- and HPLC F1-treated mice had a 17.4 ± 3.7% and 18.1 ± 2.0% relative infarct size, respectively. AAR as a percentage of the left ventricle (LV), showing the amount of endangered myocardium after MI/R injury. All animals were affected to the same extent by the operative procedure, resulting in 39.4 ± 2.0% of AAR among the groups. Each bar represents mean ± SEM.

MSC cultures derived from different tissues of three individual fetuses have a nearly identical genome-wide gene expression profile. Their gene expression profiles were also similar to that of the previously described hESC-MSCs, Hues9.E1. They were as equally proliferative as Hues9.E1 and had high levels of telomerase activity. Although the secretion by MSCs derived from different tissues, that is, limb and kidney tissues were grossly similar to that from hESC-MSCs, Hues9.E1, closer examination of specific proteins by western blot hybridization (Fig. 6B) demonstrated that there were quantitative differences in the protein composition. Consistent with this observation, secretion from the fetal MSCs significantly reduced infarct size in a mouse model of MI/R injury but at a higher dosage of 150 μg instead of 3 μg per mouse. We postulate that this difference in effective dosage was due primarily to the quantitative differences in the protein composition and candidate proteins will have to be identified through systematic evaluation and validation of quantitative high throughput proteomic

analysis. We further demonstrate that consistent with our previous observations [23], the cardioprotective effect of secretion from fetal MSCs was mediated by large complexes in the range of 100 to 200 nm or possibly, microparticles.

Microparticle is a minimally defined term that encompasses a broad spectrum of secreted particles in the size range of 0.05–1 μm. Microparticles are known to be produced by numerous cell types [42–48]. Although the function of microparticles remains poorly understood, there is irrefutable evidence that microparticles have important functions. As summarized by many recent reviews [46–57], microparticles are definitively associated with many diseases such as cancer, atherosclerosis and cerebral ischemia. However, the specific role of microparticles in disease process and/or progress remains to be determined. As some microparticles carried biologically active materials including proteins and RNAs that can be transferred between cells, it is likely that microparticles would have an impact either positively or negatively on a disease process and/or progress. This has led to suggestions that microparticles could be exploited as potential therapeutic vectors [58]. However, the broad definition of microparticles which encompasses all secreted membrane vesicles to include the more defined exosomes (50–100 nm), microvesicles (100–1000 nm), ectosomes (50–200 nm), membrane particles (50–80 nm), exosome-like vesicles (20–50 nm) and apoptotic vesicles (50–500 nm) [24] has impeded our understanding of microparticles. Other than exosomes which are the most stringently defined microparticles to date, the major distinguishing parameter for these different classes of microparticles is their size.

In this report where we not only demonstrated directly for the first time that purified microparticles in MSC secretion were cardioprotective, we also purified these microparticles by size exclusion on an HPLC which is currently one of the most precise methods for size fractionation of molecules or particles. The purified microparticles were determined by dynamic light scattering analysis to be a population of homogenously sized particles with a hydrodynamic radius of 50–65 nm. This particle size which translated into an approximate diameter of 100–130 nm was consistent with our previous observation that filtration through a membrane with an MW cutoff of 1000 kDa caused the filtrate to lose its cardioprotective function while that part of secretion retained by the same filter or the retentate was cardioprotective [23]. A membrane with an MW cutoff of 1000 kDa has a 100-nm nominal pore size according to the manufacturer (Pall Corp. http://www.pall.com/). This essentially meant that the filtrate contained particles with diameter of less than 50–100 nm and these <50–100 nm particles were not cardioprotective. On the other hand, the retentate containing particles >50–100 nm was cardioprotective. Together, this study and our previous study [23] demonstrated that cardioprotection by the secretion of hESC-MSCs and fetal MSCs was similarly mediated by microparticles with diameters of >50–100 nm. Our previous study also suggested that secretion devoid of the microparticles but containing all other secreted proteins as represented by the CM filtrate through a membrane with an MW cutoff of 1000 kDa was not cardioprotective [23]. This observation of no cardioprotective activity could therefore be extrapolated to HPLC fraction 2 to 4 where there were no microparticles and the secreted proteins were eluted according to the size exclusion principle, and were therefore soluble.

The involvement of microparticles in mediating the paracrine effect of MSC transplantation on tissue repair represents a radical shift in our current understanding of the paracrine effect of MSC which hitherto has been limited to cytokine, chemokine or growth factor-mediated extracellular signaling [10].

Together our report demonstrated that MSCs derived from fetal tissues are a viable alternative to human ESCs as a tissue source of highly proliferative MSCs that produces cardioprotective secretion. A single fetal tissue could potentially generate 10^{16} to 10^{19} MSCs. A 10^9 cell culture secretes 100 mg protein. We have previously shown that the effective cardioprotective dose for a pig model of MI/R injury was 10 mg protein per 60–70 kg pig [23]. Therefore, a single fetal tissue could potentially generate sufficient MSCs to produce 10^{8-11} doses. Together with the relatively simple purification of these microparticles to a population of homogenously sized microparticles, the use of secretion from fetal MSCs represents a viable strategy to address an urgent unmet therapeutic need for treating MI/R injury [59].

5. Disclosure statement

No potential conflict of interest.

Acknowledgments

We thank J.J. Chee (KKH) for her help in patient recruitment and tissue harvest, and Jayanthi Padmanabhan (BTI) for technical assistance in preparing the secretion.

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PAPER THREE

Characterizing the Biological Potency of MSC Exosome by Cellular and Biochemical Validation of Its Proteome

Characterizing the biological potency of MSC exosome by cellular and biochemical validation of its proteome.

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Running tittle: Biological potency of MSC exosome

Abstract

Exosomes secreted by human ESC derived mesenchymal stem cells (hESC-MSCs) were recently found to reduce infarct size in a mouse model of myocardial ischemia reperfusion injury. A requisite for elucidating the underlying mechanism is an understanding of the biological potency in these exosomes. Here we profiled the proteome of MSC exosome to identify 857 proteins that together suggest a potential to drive a diverse spectrum of cellular and biochemical activities. As an illustration and validation of this diverse potential, hESC-MSC exosomes were determined to contain glycolytic enzymes capable of generating ATP and NADH or increasing glycolysis in vitro and in vivo, 20S proteasome that hydrolyzed small peptides, CD73 that activated adenosine-mediated signaling pathway to phosphorylate ERK and AKT in cells, and CD59 that inhibited complement-mediated cell lysis. The presence of this diverse array of biological activities known to be critical in ameliorating tissue injury and facilitating tissue repair provides candidate mechanisms for cardioprotective effect of exosome from hESC-MSC.

Keywords: Mesenchymal Stem Cells, exosome, proteome, glycolysis, proteasome, ecto- 5' nucleotidase, complement-mediated cell lysis

Introduction

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.

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 857 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 and verify 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.

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.

LC MS/MS

Proteins in two ml of dialyzed exosomes were reduced, alkylated and tryptic digested as described[13]. 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 ml/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.

Antibody array

500 µl of non-conditioned media and exosomes 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.

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.

Enzymatic 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.

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.

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 ºC and fluorescense intensity at Ex/Em= 380/460 nm was monitored for 2 hours.

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.

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).

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).

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.

Results

Proteomic profiling of exosome

Exosomes were purified from culture medium conditioned by HuES9.E1, a human ESC-derived mesenchymal stem cells[14] by HPLC as previously described[9, 10]. Proteomic profiling using mass spectrometry and antibody approaches were performed as previously described[9-11]on 3 independently prepared batches of HPLC-purified exosomes. A total of 857 proteins were detected and these proteins were denoted by their gene symbol to facilitate analysis (Table 1). Of these, 320 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 1). Also consistent

with the endosomal origin of exosomes, we detected the presence of endosome-associated proteins such as Alix (PDCD6IP) and Rab (Table 1).

Computational analysis of exosome proteome

To better understand the biological significance of the proteins in the exosomes, functional clustering of the 857 proteins into biological processes was performed using PANTHER (Protein ANalysis THrough Evolutionary Relationships) analytical software[15, 16]. 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 857 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, 17] 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[18] 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 419 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 857 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

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, GAPDH, PGK, and PKm2 that generate either ATP or NADH 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[19] which catalyses the commitment to glycolysis. They are thought to be responsible for the high glycolytic rate or "Warburg effect" in cancer cells[20]. 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[21], 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 $\pm$ 28.8% or 55.8 $\pm$ 16.5% in 15 to 30 minutes of exposure to exosomes respectively, possibly through increased glycolysis.

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 [22]. 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[23] 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.

Exosome phosphorylated ERK and AKT via CD73 (ecto-5'-ectonucleotidase, NT5E)

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[24]. CD39 hydrolyzes extracellular ATP and ADP to AMP which is then degraded to adenosine by ecto-5'-nucleotidase. Adenosine has a half-life of less than 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)[25].

CD73 was found to be present in the MSC exosomes by mass spectrometry analysis and confirmed by immunoblotting (Figure. 5A). The enzyme activity was 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, C). 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 that antagonized A1, A2A, A2B, and A3 receptors[26].

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[27].

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[28], 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.

Discussion

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 857 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[29] and the presence of these proteins is probably a reflection of the biogenesis of exosomes. It was previously observed that exosomes from different cell sources carry a common set of proteins, many of which are reflective of their biogenesis[30]. 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[31], genetic exchange[32], immune responses[33, 34] and tumor metastasis[33-38]. 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, 39]. 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[19], 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[40]. 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[41] 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[42, 43] or cardiovascular disease[44-46].

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[47]. Therefore, the activation of adenosine receptor which has been shown to be cardioprotective[47, 48]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[49-51].

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. 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.

Acknowledgements

We gratefully acknowledge Kong Meng Hoi and Eddy Tan at the Bioprocessing Technology Institute (BTI) for helping in the purification of the exosomes and Jayanthi Padmanabhan and Jeremy Lee (BTI) for the preparation and concentration of the conditioned medium.

Conflicts of interest

No conflicts of interest

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Figures

Figure 1: Intersection of the 739 proteins previously identified in MSC conditioned medium versus the 857 proteins identified in purified exosomes.

Figure 1

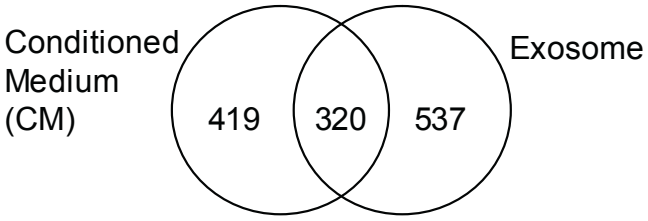


Figure 2. Proteomic analysis of exosome proteins. The 857 gene products in exosomes were functionally clustered into 32 over-represented and three under-represented biological processes ($p<0.001$). The 419 proteins found in the conditioned medium (CM) but not in the exosome were functionally clustered 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.

Figure 2

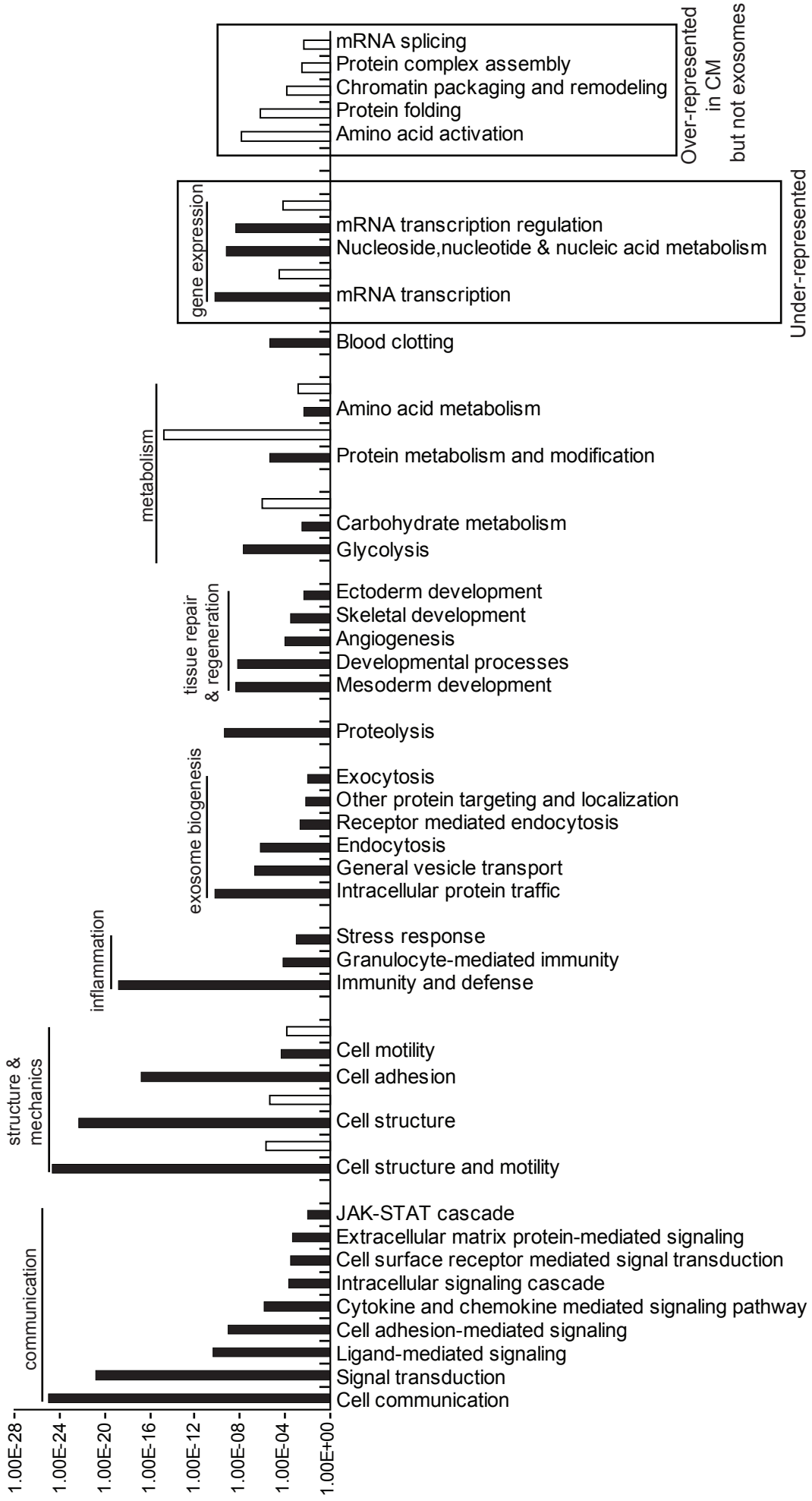
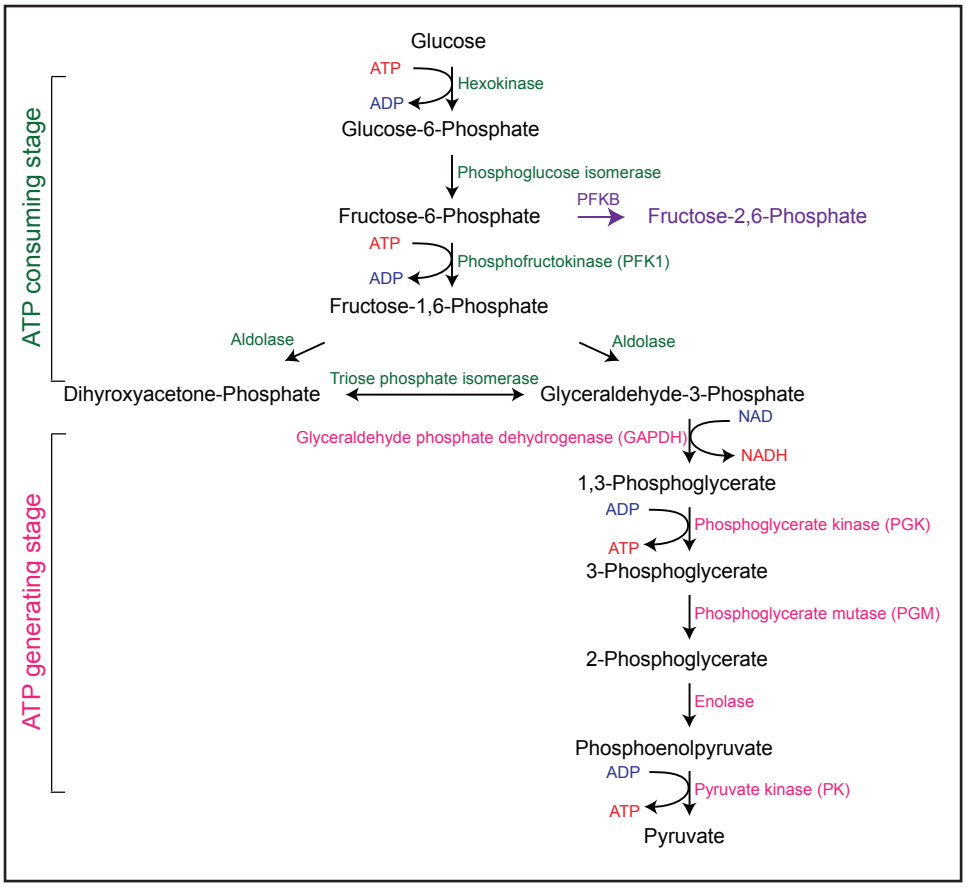
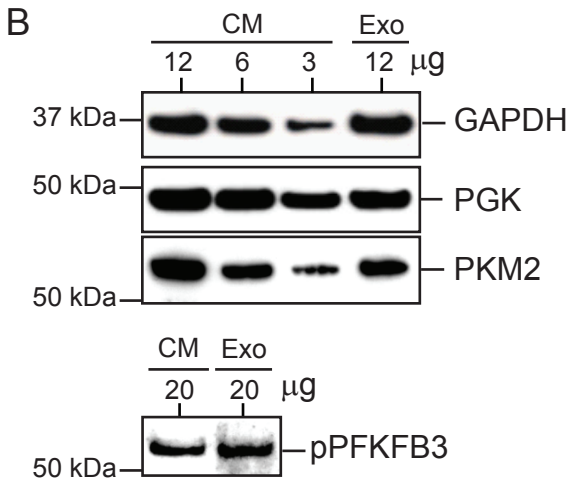
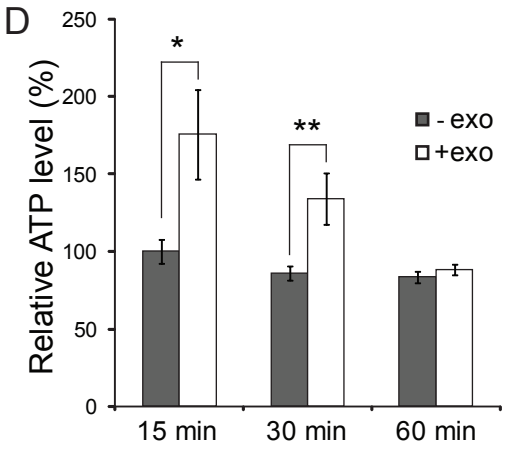

1.00E-28
1.00E-24
1.00E-20
1.00E-16
1.00E-12
1.00E-08
1.00E-04
1.00E+00
communication
Cell communication
Signal transduction
Ligand-mediated signaling
Cell adhesion-mediated signaling
Cytokine and chemokine mediated signaling pathway
Intracellular signaling cascade
Cell surface receptor mediated signal transduction
Extracellular matrix protein-mediated signaling
JAK-STAT cascade
structure & mechanics
Cell structure and motility
Cell structure
Cell adhesion
Cell motility
inflammation
Immunity and defense
Granulocyte-mediated immunity
Stress response
exosome biogenesis
Intracellular protein traffic
General vesicle transport
Endocytosis
Receptor mediated endocytosis
Other protein targeting and localization
Exocytosis
Proteolysis
tissue repair & regeneration
Mesoderm development
Developmental processes
Angiogenesis
Skeletal development
Ectoderm development
metabolism
Glycolysis
Carbohydrate metabolism
Protein metabolism and modification
Amino acid metabolism
Blood clotting
gene expression
mRNA transcription
Nucleoside,nucleotide & nucleic acid metabolism
mRNA transcription regulation
Under-represented
Amino acid activation
Protein folding
Chromatin packaging and remodeling
Protein complex assembly
mRNA splicing
Over-represented in CM but not exosomes

Figure 3: Exosome regulates glycolysis. (A) Schematic diagram of glycolysis. (B) 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. (C) 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. (D) 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

Figure 3


A
ATP consuming stage
ATP generating stage
Glucose
ATP
Hexokinase
ADP
Glucose-6-Phosphate
Phosphoglucose isomerase
Fructose-6-Phosphate
PFKB
Fructose-2,6-Phosphate
ATP
Phosphofructokinase (PFK1)
ADP
Fructose-1,6-Phosphate
Aldolase
Aldolase
Triose phosphate isomerase
Dihyroxyacetone-Phosphate
Glyceraldehyde-3-Phosphate
NAD
Glyceraldehyde phosphate dehydrogenase (GAPDH)
NADH
1,3-Phosphoglycerate
ADP
Phosphoglycerate kinase (PGK)
ATP
3-Phosphoglycerate
Phosphoglycerate mutase (PGM)
2-Phosphoglycerate
Enolase
Phosphoenolpyruvate
ADP
Pyruvate kinase (PK)
ATP
Pyruvate


B
CM
Exo
12
6
3
12
µg
37 kDa
GAPDH
50 kDa
PGK
50 kDa
PKM2
CM
Exo
20
20
µg
50 kDa
pPFKFB3


D
Relative ATP level (%)
250
200
150
100
50
0
*
**
- exo
+exo
15 min
30 min
60 min

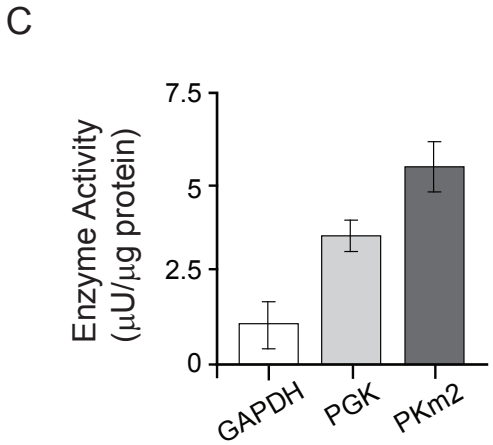

C
Enzyme Activity
(µU/µg protein)
7.5
5
2.5
0
GAPDH
PGK
PKm2

Figure 4: 20S proteasome in exosome. (A) Western blot analysis of MSC conditioned medium (CM) and exosome (Exo) using an antibody specific for PMSA 1-7 peptides. (B) 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

Figure 4

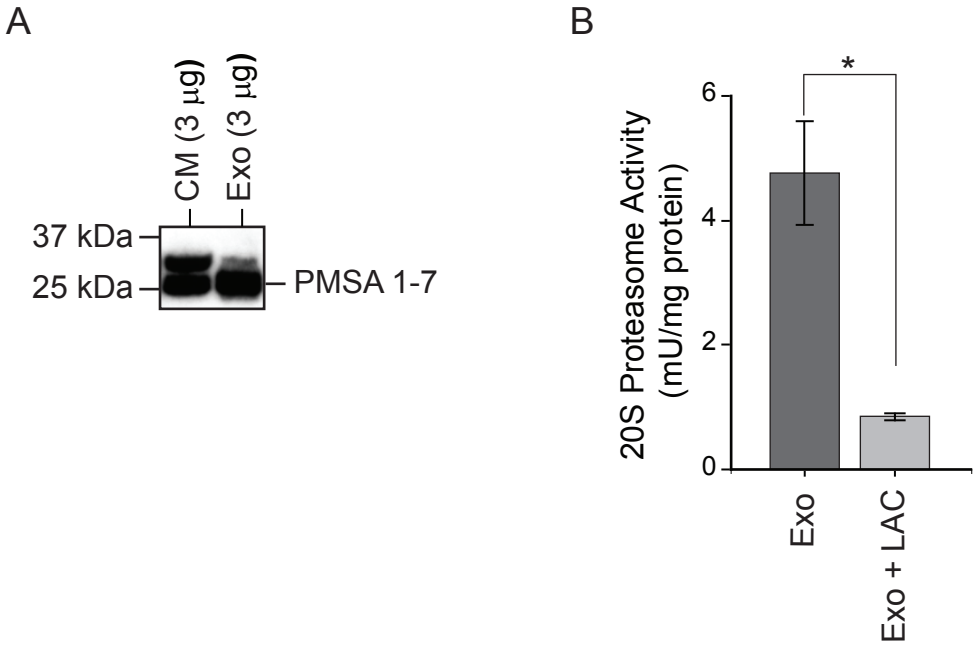

A
CM (3 µg)
Exo (3 µg)
37 kDa
25 kDa
PMSA 1-7
B
20S Proteasome Activity
(mU/mg protein)
6
4
2
0
*
Exo
Exo + LAC

Figure 5: Exosome phosphorylated ERK and AKT via NT5E (ecto-5'-ectonucleotidase CD73). (A) Western blot analysis of MSC CM and exosome for CD73 using a specific antibody. (B) CD73 activity in conditioned medium (CM) and exosomes (Exo) was measured by the production of phosphate ion from the hydrolysis of AMP. (C) 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.

Figure 5

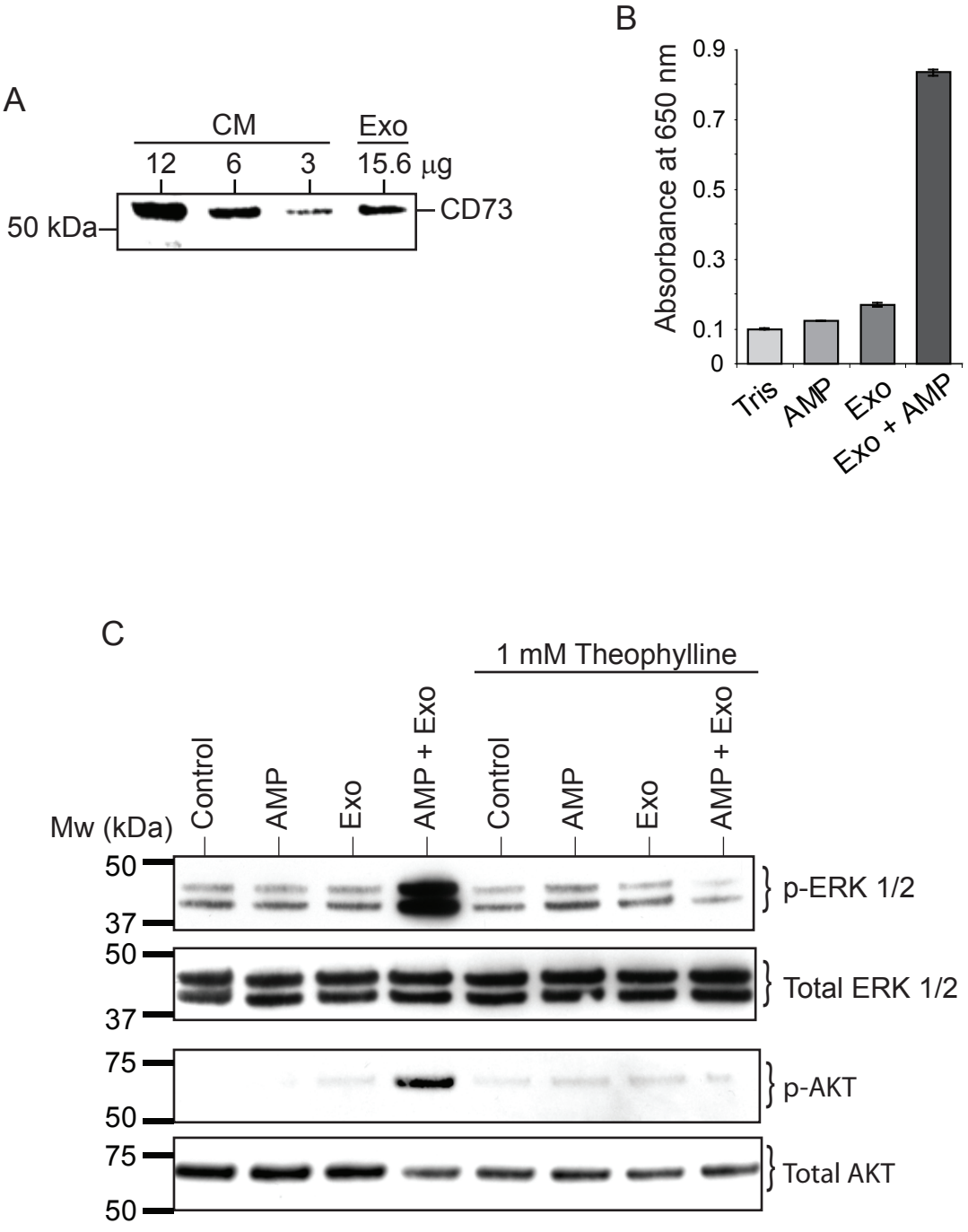

A
CM
Exo
12
6
3
15.6 µg
50 kDa
CD73
B
Absorbance at 650 nm
0.9
0.7
0.5
0.3
0.1
0
Tris
AMP
Exo
Exo + AMP
C
1 mM Theophylline
Control
AMP
Exo
AMP + Exo
Control
AMP
Exo
AMP + Exo
Mw (kDa)
50
37
50
37
75
50
75
50
p-ERK 1/2
Total ERK 1/2
p-AKT
Total AKT

Figure 6: Exosome inhibited the formation of membrane attack complex (MAC). (A) Western blot analysis of MSC conditioned medium (CM) and exosome (Exo) using a CD59-specific antibody. (B) 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 6

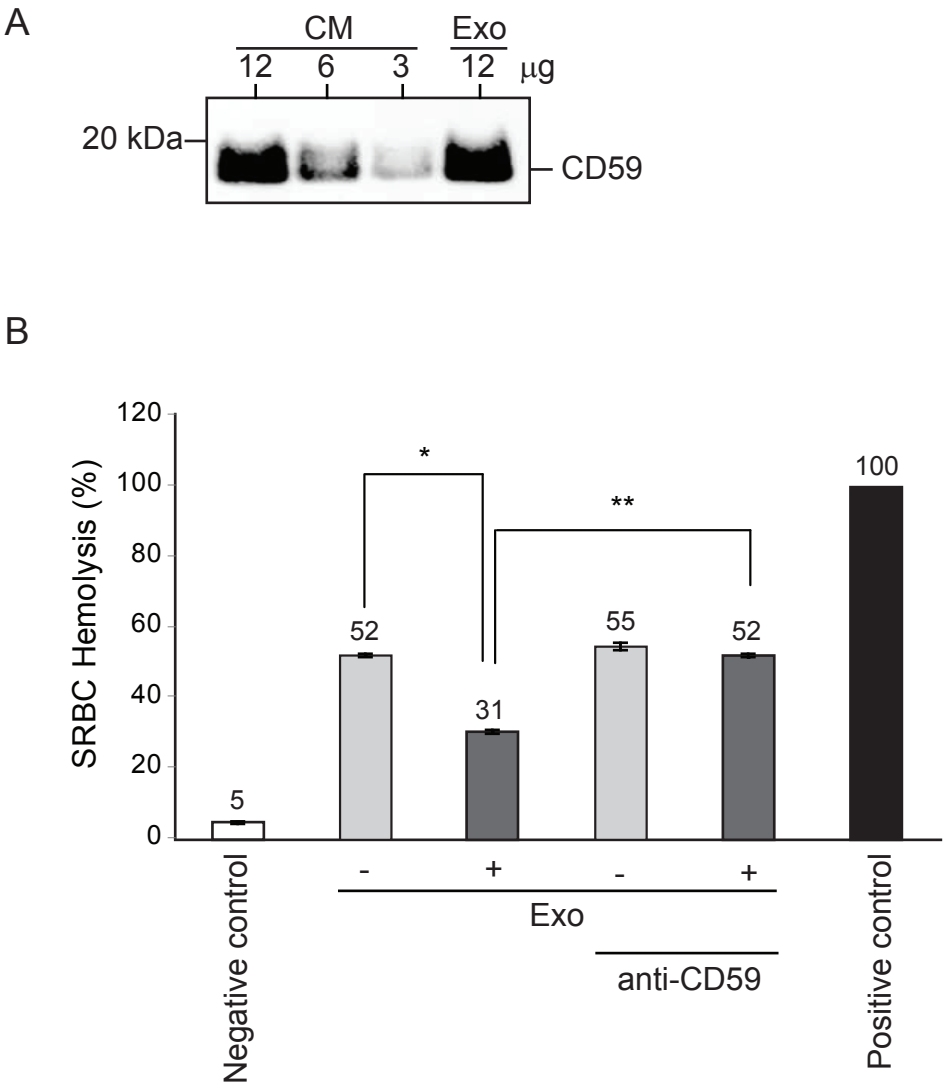

A
CM
Exo
12
6
3
12
μg
20 kDa
CD59
B
SRBC Hemolysis (%)
120
100
80
60
40
20
0
*
**
5
52
31
55
52
100
-
+
-
+
Exo
anti-CD59
Negative control
Positive control

Table 1

A2M	C11orf59	COPS4	FBXW8	HNRNPA1	KRT16	MMP3	PRR4	RPS3	TGFB2
ABI3BP	C1orf78	COPS8	FEN1	HP	KRT17	MOS	PRSS23	RPS4X	TGFBI
ACAA2	C1R	CPS1	FER1L3	HPX	KRT18	MPO	PSMA1	RPS5	TGM2
ACAT2	C1S	CREG1	FGA	HRSP12	KRT19	MPZL1	PSMA2	RPSA	TGOLN2
ACLY	C20orf114	CRIPT	FGB	HSP90AA1	KRT2	MRC2	PSMA3	RRAS2	THBS1
ACSL1	C3	CRTAP	FGF16	HSP90AB1	KRT27	MSN	PSMA4	RTN4	**THBS2**
ACTA1	C5orf24	CSF1	FGF18	HSP90B1	KRT28	MXRA5	PSMA5	RUVBL1	THY1
ACTA2	C9orf19	CSF2	FGF19	HSPA1A	KRT3	MYADM	PSMA6	S100A11	TIMP1
ACTB	C9orf91	CSF3	FGFRL1	HSPA1L	KRT4	MYCBPAP	PSMA7	S100A13	TIMP2
ACTG2	CACNA2D1	CSPG4	FGG	HSPA5	KRT5	MYH14	PSMB1	S100A8	TIMP3
ACTN1	CACNA2D4	CST4	FLG2	HSPA6	KRT6A	MYH9	PSMB10	S100P	TKT
ACTN2	CALR	CTA-221G9.4	FLJ13197	HSPA8	KRT6B	MYL6B	PSMB2	SAA4	TLN1
ACTN3	CAND1	CTBP2	FLJ22184	HSPB1	KRT6C	MYO1C	PSMB3	SASS6	TMBIM1
ACTN4	CAP1	CTNNA1	FLJ32784	HSPD1	KRT7	NBL1	PSMB4	SCAMP3	TMED10
ACTR1A	CAPNS1	CTNNA2	FLNA	HSPG2	KRT72	NEFH	PSMB5	SCGB2A1	TMED9
ACTR2	CAPZA1	CTNNB1	FLNB	HTRA1	KRT73	NEK10	PSMB6	SCYE1	TMEM16B
ACTR3	CASP14	CTNND1	FLNC	HYI	KRT74	NID1	PSMB7	SDC1	TMEM2
ADAM10	CAT	CTSG	FLOT1	**ICAM1**	KRT76	NLRP8	PSMB8	SDC2	TMEM47
ADAM9	CAV1	CXCL16	FLOT2	ICAM5	KRT77	NME1	PSMB9	SDC4	TMEM51
ADAMTS12	CCDC129	CXCL2	FLT1	IDH3B	KRT78	NOMO1	PSMC5	SDCBP	TNC
AEBP1	CCDC64B	CXorf39	FN1	IFITM2	KRT79	NRAS	PSMD11	SEC14L4	TNFRSF11B
AFM	CCL2	CYBRD1	FREM3	IFNG	KRT8	NRG2	PSMD14	SEMA5A	TNFRSF12A
AGRN	CCL20	DBF4B	**FST**	IFRD1	KRT80	NRLN1	PSMD6	SEPT2	TNFRSF1A
AHCY	CCL28	DCD	FTL	IFT140	KRT84	NRP1	PSMD7	SEPT7	TNFSF18
AHNAK2	CCL7	DCHS2	FUCA2	IGF2R	KRT9	NT5E	PTGFRN	SERINC5	TNFSF5
AHSG	CCR4	DCLK2	GALNT5	IGFBP3	LACRT	NTF5	PTK7	SERPINA1	TPBG
AKR1B1	CCR5	**DCN**	GANAB	IGFBP4	LAMA4	NUSAP1	PTPRK	SERPINB3	TPI1
AKR7A2	CCT5	DCTN1	GAPDH	IGFBP6	LAMB1	OBFC1	PTRF	SERPINE1	TRAP1
ALB	CCT6A	DECR1	GAPDHS	**IGFBP7**	LAMC1	ODZ3	PTTG1IP	SERPINE2	TREM1
ALCAM	CD109	DEFA1	GARS	IGHA1	LAMP1	OFD1	**PTX3**	SERPINF1	TREML2P
ALDH2	CD151	DIP2B	GAS6	IGHA2	LAMP2	OPRM1	PXDN	SFN	TRIM40
ALDH3A2	CD248	DIRAS2	GDF1	IGHG1	LAP3	OSM	PZP	SFRP1	TRIM41
ALDH6A1	CD276	DKFZp686D0972	GDF11	IGHG2	LCN1	OTC	QPCTL	SFRP4	TSN
ALDH7A1	CD44	DKK1	GDF3	IGHG4	LCN2	OXNAD1	QSOX1	SHANK3	TSNAX
ALDH9A1	CD47	DKK3	GDF5	IGHM	LDHA	OXTR	RAB10	SLAIN1	TSPAN14
ALDOA	CD59	DMBT1	GDF8	IGJ	LDHAL6B	P4HB	RAB11B	SLC16A1	TSPAN4
ALDOB	CD63	DNASE1L1	GDF9	IGKC	LDHB	PAICS	RAB14	SLC16A3	TSPAN6
ALDOC	CD81	DNPEP	GDI1	IGKV1-5	LEPRE1	PAN3	RAB15	SLC1A4	TSPAN9
ALOX12P2	CD82	DPYS	GDI2	IGL@	LGALS1	PAPPA	RAB1A	SLC1A5	TSTA3
ANG	CD9	DPYSL2	GFRA3	IGLV4-3	LGALS3	PARP10	RAB1B	SLC22A2	TTLL3
ANGPTL2	CDC2L5	DSP	GLDC	IGSF8	LGALS3BP	PARP16	RAB2A	SLC25A10	TTN
ANPEP	CDC42	DULLARD	GLUD1	IL10	LGALS8	PARVG	RAB33B	SLC25A13	TTYH3
ANXA1	CDH13	ECM1	GNA13	IL11	LGR6	PC	RAB35	SLC2A1	TUBA1A
ANXA11	CDIPT	ED1	GNAI2	IL13	LIF	PCOLCE	RAB39B	SLC2A3	TUBA1B
ANXA2	CDK5R2	EDG2	GNAL	IL15RA	LMNA	PDCD6	RAB5A	SLC38A2	TUBA1C
ANXA2P1	CEACAM8	EDIL3	GNAS	IL17B	LOC124220	PDCD6IP	RAB5B	SLC38A3	TUBB
ANXA3	CFB	EEA1	GNAT3	IL17R	LOC283523	PDGFA	RAB5C	SLC39A14	TUBB2A
ANXA4	CFI	EEF1A1	GNB1	IL19	LOC284297	PDGFC	RAB6A	SLC3A2	TUBB2C
ANXA5	CFL1	EEF1G	GNB2	IL1F9	LOC388344	PDGFRB	RAB7A	SLC44A1	TUBB3
ANXA6	CFL2	EEF2	GNB4	IL1RAP	LOC389827	PDIA3	RAB8A	SLC44A2	TUBB6

ANXA7	CFTR	EFEMP2	GNG12	IL1RAPL1	LOC442497	PEBP1	RAB8B	SLC7A10	UBA52
AP1S1	CHMP2A	EHD1	GNPDA1	IL1RL2	LOC653269	PFAS	RAC1	SLC7A5	UBB
APEH	CHST12	EHD2	GOT2	IL22RA1	LOC727942	PFKFB3	RAC2	SMARCA4	UBE1
APOA1	CITED1	EHD4	GPC1	IL23A	LOC728320	PFN1	RAD21	SMC1A	UBE2N
APOE	CLASP2	EIF4A1	GPC5	IL3	LOC728378	PFN2	RALA	SORT1	UGP2
APP	CLDN1	EMILIN1	GPI	IL5	LOC730013	PGAM2	RAN	SPACA1	UNC13B
ARF1	CLEC11A	ENG	GPR112	IL6ST	LRP1	PGD	RAP1A	SPARC	UNC45A
ARF4	CLIC1	ENO1	GREM1	IL7	LRP6	PGK1	RAP1B	SPOCK1	VAMP3
ARF5	CLIC6	ENO2	GRM2	IL8	LRRFIP2	PGLYRP2	RAP1B	SPRY4	VANGL1
ARHGAP18	CLPX	ENO3	GRM3	INHBA	**LTBP1**	PIGR	RAP2C	SPTAN1	**VASN**
ARHGAP23	CLSTN1	ENTPD4	GRM7	INHBB	LTBP2	PIP	RARRES1	SPTBN1	VAT1
ARHGDIA	CLTA	ENTPD4;LOXL2	GSN	INSR	LTF	PKM2	RASA1	SPTBN4	VCAN
ARHGEF1	CLTC	EPB41L3	GSTM1	IQGAP1	LYAR	PLAB	RASA4	SRGN	VCL
ARL6IP5	CLTCL1	EPHA2	GSTM2	ITGA11	LYZ	PLAU	RB1CC1	SRI	VCP
ARMS2	CLU	EPO	GSTM5	ITGA2	MADH4	PLEC1	RCOR2	SRPX2	VEGFC
ARPC3	CMIP	ESM1	GSTO1	ITGA3	MAMDC2	PLEKHG3	RDH5	ST6GALNAC6	VIL1
ARPC4	CNGB1	ETFB	GSTP1	ITGA4	MAP1A	PLOD1	RFTN1	STAT1	VIL2
ARPC5	COL12A1	F2R	GTPBP2	ITGA5	MAP2K6	PLOD2	RGN	STC1	VIM
ASH1L	COL14A1	**F3**	GYLTL1B	ITGAL	MAP3K1	PLOD3	RHOC	STC2	VTI1A
ASL	COL18A1	F8	GZMA	ITGAV	MARCKS	PLP2	RMND5A	STOM	VTN
ATP1A1	COL1A1	FADD	H2AFV	ITGB1	MARCKSL1	PLSCR3	RNF123	STOML3	WDR49
ATP1B3	COL1A2	FAH	H2AFX	ITGB5	MAT1A	PLTP	RNF40	STX12	WDR52
ATP2B1	COL2A1	FAM108A1	HBB	ITIH2	MBD3	PLUNC	RPL10A	STX2	WNT5A
ATP2B4	COL3A1	FAM129B	HBE1	ITIH4	MCC	PNO1	RPL12	SURF4	YBX1
ATP5A1	COL4A1	FAM29A	HDAC5	ITPR2	MCM10	PODN	RPL15	SVEP1	YWHAB
ATP5B	COL4A2	FAM3B	HERC5	JUP	MDH1	POLN	RPL18	SYT1	YWHAE
ATP8B3	COL4A3	FAM64A	HGF	KIAA0146	MDH2	POSTN	RPL23	SYT9	YWHAG
ATRN	COL5A1	FAM71F1	HGFR	KIAA0256	ME1	POTE2	RPL29	TAAR2	YWHAQ
ATXN1	COL5A2	FAP	HISPPD2A	KIAA0467	MECP2	PPIA	RPL35A	TAGLN	YWHAZ
AXL	COL6A1	FASN	HIST1H2AE	KIAA1881	MFAP4	PPIB	RPLP0	TALDO1	ZBTB4
BASP1	COL6A2	FAT	HIST1H2BA	KPNB1	MFGE8	PPME1	RPS10	TAS2R60	ZNF134
BDNF	COL6A3	FAT2	HIST1H2BL	KRT1	MFSD2	PPP1CC	RPS16	TCN1	ZNF503
BGN	COL7A1	FAT4	HIST1H4H	KRT10	MIF	PRDM16	RPS18	TF	ZNF614
BHMT2	COMP	FBLN1	HIST2H2BE	KRT13	MMP1	PRDX1	RPS2	TFG	
BRMS1	COPB1	FBN1	HLAA	KRT14	MMP10	PRDX6	RPS24	TFRC	
BSG	COPS3	FBN2	HMGCS2	KRT15	**MMP2**	PRNP	RPS27A	TGFB1	

Regular font	Identified by LC MS/MS				
Underline	Identified by antibody arrays				
Bold and Underline	Identified by both LC MS/MS and antibody arrays				
Grey shade	Identified by LC MS/MS and was found to be present in at least 50% of exosomes characterized[2]				

Table 1: Proteomic profile of 3 independently prepared exosomes as determined by LC MS/MS and antibody arrays.

PAPER FOUR

Exosomes Target Multiple Mediators To Reduce Cardiac Injury

Exosomes target multiple mediators to reduce cardiac injury

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Total Word Count: 4.226

Journal Subject Heads: myocardial infarction, ischemic biology – basic studies, apoptosis

Abstract

Background

Paracrine factors secreted by stem cells are believed to mediate therapeutic actions of current cell therapy for myocardial infarction. The usage of stem cell secretion represents an attractive 'off-the-shelf' therapeutic alternative for cell therapy. Here we describe both therapeutic and mechanistic actions of mesenchymal stem cell (MSC)-derived exosomes in myocardial ischemia/reperfusion (I/R) injury.

Methods and Results

Mice underwent 30 minutes ischemia, followed by reperfusion. Compounds were administered 5 minutes before reperfusion. Cardiac function and geometry were assessed using 9.4T mouse-MRI and invasive PV-loop recordings. Purified exosomes reduced infarct size to the same extent as MSC-conditioned medium when compared to saline treatment (38.5±1.8% vs. 21.3±2.2%, $p<.001$). *Ex vivo* I/R experiments showed similar infarct size reduction compared to the *in vivo* situation, suggesting that exosomes directly improve myocardial survival. Post-infarct heart tissue analysis revealed that exosomes enhanced survival via Akt and GSK-3β phosphorylation. Apoptosis was also reduced as shown by reduced phosphorylated c-JNK levels and reduced TUNEL-positive staining. Exosomes prevented energy depletion (ADP/ATP) and improved redox state (NAD+/NADH) in the myocardium after I/R. Furthermore, exosome treatment reduced infarct-induced inflammation after ischemia/reperfusion. Compared to saline treatment, exosomes prevented left ventricular dilatation (end-diastolic volume: 81±7 vs. 58±4 μL, $p=.021$) and preserved systolic and contractile performance 28 days after I/R.

Conclusions

Systemic administration of MSC-derived exosomes 5 minutes prior to reperfusion reduces infarct size and preserves cardiac function and geometry. Exosome treatment promotes pro-survival signaling, enhances energy balance and reduces secondary inflammation. Hence, MSC-derived exosomes are a potential candidate as an adjunctive for reperfusion therapy in patients with myocardial infarction.

Keywords: myocardial infarction, reperfusion, paracrine hypothesis, exosomes, mesenchymal stem cells

The socio-economic burden of myocardial infarction (MI) and related complications (e.g. heart failure) is increasing in Western societies. Recent advances in (interventional) cardiology have resulted in timely and optimized coronary flow through the culprit artery. Subsequently, more patients survive the initial infarction, but have worsened cardiac performance and increased infarct related morbidity.[1] The increase in morbidity after MI triggered the search for adjunctive therapeutics to further limit excessive tissue loss and enhance cardiac performance. Stem cell therapy has been shown to have great potential in the treatment of patients with MI.[2] More interestingly, it is widely accepted that the observed therapeutic effects are mediated by stem cell secretion. This so called 'paracrine hypothesis' has gained much attention and is supported by recent experimental data[3]. It has been shown that MSC-CM enhance cardiomyocyte and/or progenitors survival after hypoxia-induced injury.[4-8] Furthermore, MSC-CM induce angiogenesis in the infarcted myocardium.[5, 6, 9] We have shown in both murine and porcine models of myocardial I/R that MSC-CM reduces infarct size.[10] Interestingly, we found that the therapeutic action was irrespective of the cell source for the MSCs; human embryonic stem cell-derived MSCs were not superior to those derived from distinct fetal cell sources (e.g. limb, kidney). High performance liquid chromatography (HPLC) and dynamic light scatter (DLS) analyses revealed that both MSC sources secreted similar cardioprotective microparticles with a size ranging from 50-65 ηm.[11] Purification of these microparticles resulted in the identification of exosomes as the cardioprotective factor in MSC-CM[12].

Exosomes are bi-lipid membrane vesicles with a diameter of 50-100 ηm. They are secreted by various cell types and are involved in immune responses, intercellular communication and antigen presentation.[13] Multivesicular bodies store the exosomes within the cell and release them upon fusion with the plasma membrane. The identification of exosomes as the cardioprotective factor in MSC secretion make it an potential therapeutic tool in treating

myocardial reperfusion injury. In contrast to cell-based therapy, MSC-derived exosomes provide an 'off-the-shelf' therapeutic. Furthermore, exosomes are potentially safer compared to cell therapy since they are non-viable. In addition, the biologic-based approach of exosomes may reduce the manufacturing costs. Although we previously described that exosomes reduce infarct size in mice,[12] the functional consequence and the mechanism for its cardioprotective actions remain unknown.

In the present study, we describe the functional improvement after exosomes treatment in myocardial I/R. In addition, we identified for the first time several pathways involved in myocardial I/R injury that are targeted simultaneously by MSC-derived exosomes to exert cardioprotective actions.

Methods

Animals and Experimental Design

Male C57Bl6/J mice (10-12 wks old, 25-30 g) received standard diet and water *ad libitum*. Saline or exosomes were administered intravenously via the tail vein, 5 minutes before reperfusion. Myocardial infarction was induced by temporary left coronary artery ligation, just below the left atrial appendage as described previously.[14] Where possible, recommendations from the National Heart Lung and Blood Institute (NHLBI) Working Group on the Translation of Therapies for Protecting the Heart from Ischemia[15] were applied; Digital photos of infarcts were encrypted before being analyzed by the researcher. Heart function and geometry assessment was done by a technician blinded to treatment. All animal experiments are performed in accordance with the national guidelines on animal care and with prior approval by the Animal Experimentation Committee of Utrecht University.

Myocardial I/R Injury In Vivo

Mice were anesthetized with a mixture of Fentanyl (Jansen-Cilag) 0.05 mg/kg, Dormicum (Roche) 5 mg/kg and medetomidine 0.5 mg/kg through an intraperitoneal injection. Core body temperature was maintained around 37°C during surgery by continuous monitoring with a rectal thermometer and automatic heating blanket. Mice were intubated and ventilated (Harvard Apparatus Inc.) with 100% oxygen. The left coronary artery (LCA) was ligated for 30 minutes with an 8-0 Ethilon (Ethicon) with a section of polyethylene-10 tubing placed over the LCA. Ischemia was confirmed by bleaching of the myocardium and ventricular tachyarrhythmia. In sham operated animals the suture was placed beneath the LCA without ligating. Reperfusion was initiated by releasing the ligature and removing the polyethylene-10 tubing. Reperfusion of the endangered myocardium was characterized by typical hyperemia in the first few minutes. In a subgroup of mice, a piece of the loosened suture was left in place to

determine the area-at-risk (AAR) during termination. The chest wall was closed and the animals received subcutaneously Antisedan (Pfizer) 2.5 mg/kg, Anexate (Roche) 0.5 mg/kg and Temgesic (Schering-Plough) 0.1 mg/kg.

Data Supplement

The online Data Supplement contains detailed information on exosome purification, Langendorff I/R injury, infarct size calculation, MRI measurements, invasive LV pressure measurements, immunohistochemistry, flow cytometry (cytokine and survival kinases) and ELISA for ADP/ATP and NAD+/NADH.

Statistical Analysis

Data are represented as Mean±SEM. One-way ANOVA with post-hoc 2-sided Dunnett *t*-test adjustment (saline was set as control) was used for infarct size comparison between the groups. Non-parametric t-test was used for 2 group comparisons. All statistical analyses were performed using SPSS 15.1.1. and $p < 0.05$ was considered significant. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Intact Exosomes Reduce Myocardial I/R Injury In Vivo and Ex Vivo

Thirty minutes ischemia followed by 24 hours reperfusion in saline treated animals resulted in 39±1.8% infarction within the AAR (IS/AAR; Figure 1A). A single i.v. bolus of exosomes 5 minutes prior to reperfusion reduced infarct size to 45% (21±2.2% IS/AAR; $p<0.001$). We performed *ex vivo* I/R experiments in the Langendorff setup, to study whether exosomes exert their therapeutic effect via blood cells or via direct survival enhancement of myocardial cells. Three hours reperfusion with normal buffer after 30 minutes ischemia resulted in 49±5.3% IS/AAR. Surprisingly, *ex vivo* exosome treatment reduced infarct size to the same extent as in the *in vivo* situation (23±1.5% IS/AAR; $p=0.002$). This suggests that exosomes directly target myocardial cells to reduce I/R injury. It is postulated that exosomes fuse with target cells in order to release their content.[13] To test this hypothesis, we disrupted the exosomes by vigorous agitation (2x30 sec) in a homogenizer (Precellys®24, Bertin Technologies, France). As a result, disrupted exosomes failed to reduce infarct size *in vivo* (37±3.0% IS/AAR; $p=.994$). In our experiments, the extent of endangered myocardium was similar in all groups (mean AAR/LV = 40±1.3%; Figure 1B).

Exosome Treatment Prevents Left Ventricular Dilatation and Improves Cardiac Performance

We performed serial cardiac MRI measurements to assess both left ventricular (LV) function and geometry after I/R injury. There were no differences in LV function and dimensions at baseline. The infarct size reduction in exosome treated animals translated into significant preservation of both end-diastolic and –systolic volume (EDV, ESV) during follow-up. In addition, ejection fraction was significantly improved after exosomes treatment at all time

points (Figure 2A-C). Wall thickness (WT) of the infarcted area (free wall) was equally increased in saline and exosomes treated animals 1 day after reperfusion (Figure 2D), presumably caused by tissue edema. However, exosome treatment resulted in decreased thinning of the infarct area during scar maturation. This finding is in line with the infarct size reduction seen after exosome treatment. There were no differences in WT in the remote myocardium between the groups (Figure 2E). The higher extent of viable tissue after exosome treatment also significantly improved systolic thickening of the infarcted area at all time points (Figure 2F), and in the remote myocardium 28 days after infarction (Figure 2G).

We performed invasive pressure-volume (PV) loop recording to determine the hemodynamic consequences of reduced dilatation and improved systolic performance after exosome treatment. In support of increased viability of the endangered myocardium, we observed higher contractility and relaxation in exosome treated mice 28 days after infarction. In addition, preserved LV geometry was associated with reduced end-diastolic pressure (EDP) in exosome treated animals (Table 1).

Exosome Treatment Reduces Inflammation After I/R

Myocardial I/R is characterized by accentuated tissue and systemic inflammation.[16] In line with reduced cardiac damage upon exosome treatment, we observed reduced neutrophil infiltration, but not a significant macrophage influx, in exosome treated hearts compared to saline treatment (Figure 3A and B). In patients after MI , high peripheral white blood cell (WBC) count is associated with larger infarct size, worse cardiac performance and poor clinical outcome.[17-21] In concordance with clinical studies, our experiments revealed that infarct size reduction and improved cardiac performance in exosome treated animals is associated with reduced WBC count after I/R injury (Figure 3C). The reduction of neutrophil

infiltration and peripheral WBC count also suggest that exosomes are probably non-immunogenic.

Exosome Treatment Phosphorylates Akt/GSK3 Pathway, Inhibits c-JNK Signaling and Reduces Apoptosis After I/R

Paracrine actions of MSC secretion is shown to be mediated via enhanced phosphorylation of survival pathways, especially of PI3K/Akt pathway.[3-7] In our study, exosome treatment induced Akt and GSK3 phosphorylation, it was already significant at 1 hour reperfusion (Figure 4A and B). ERK1/2 phosphorylation has been shown to be protective as well in myocardial I/R injury.[22] In our experiments, however, ERK1/2 phosphorylation was not altered after exosome treatment (Figure 4C). These findings suggest that MSC-derived exosomes specifically target Akt and GSK3 pathways to induce pro-survival effects. Phosphorylation of c-JNK is a known activator of pro-apoptotic signaling in myocardial I/R injury. As shown in Figure 4D, exosome treatment also reduced deleterious phosphorylation of c-JNK at 1 hour reperfusion. To support the reduction of apoptotic signaling, we performed a TUNEL staining on heart sections 1 day after reperfusion. Indeed, apoptosis was reduced after exosome treatment compared to saline treated mice (Figure 4E).

Exosome Treatment Restores Energy Depletion and Redox State

Myocardial I/R injury results in rapid depletion of ATP and increased oxidation of NADH into NAD+.[22] We studied these processes at very early time points to demonstrate the fast mode of action of exosome treatment after I/R injury. Figure 5A and B demonstrate that

exosome treatment just prior to reperfusion restores both ADP/ATP and NAD+/NADH levels 30 minutes after reperfusion.

Discussion

Previous studies have shown that MSC transplantation is successful in improving cardiac function after infarction. Nevertheless, a biologic-based approach may be preferable for it to overcome the limitations of cell-based therapy. MSC-derived-exosomes are a) non-viable, b) relatively easy to scale up and manufacture, c) non-immunogenic thus facilitating d) an 'off-the-shelf' therapeutic option in acute MI. So far, the vast majority of research examining the paracrine actions of MSCs has focused on single mediators within the secretion. However, the pathophysiological changes after myocardial I/R are complex and multifactorial involving many cell types (resident and migrating cells) and many intracellular signaling cascades. For this reason, it is likely that MSC secretion targets multiple mediators of injury to exert therapeutic effects. We have shown that exosomes are robust and reproducible candidates for adjunctive therapy in MI.[11, 12] Exosomes have shown to be promising as vaccination vehicles and have great potential for cancer treatment.[13, 23-25] For this reason, we believe that exosomes also serve as a drug delivery platform for the cardioprotective factors in MSC secretion.

In the present study, we have demonstrated that intravenous MSC-derived exosome administration just 5 minutes prior to reperfusion reduces infarct size with approximately 45%. Our clinically relevant model shows that MSC-derived exosomes have the potential to be successful in acute MI. Patients suffering from an acute myocardial infarction may benefit from MSC-derived exosomes when administered intravenously in the ambulance or in the emergency room before undergoing reperfusion therapy.

Our results showed that, as a consequence of reduced infarct size, MSC-derived exosome treatment resulted in a significant preservation of LV geometry and both systolic and diastolic performance during long-term follow-up. Although infarct size was reduced, exosome treatment did not reduce reperfusion-induced edema as shown by equal increase of thickness

of the free wall (infarct area) 1 day after reperfusion. The similar extent of tissue edema at day 1 in the MRI studies suggests that the amount of endangered myocardium did not differ between the groups. Ex vivo I/R experiments revealed that exosomes directly target myocardial cells to enhance viability. In addition, it appeared that the therapeutic action required intact exosomes. The latter is supported by previous experiments in which we showed that exosomes have shown to fuse with cardiomyocytes.[26] Disruption of the bi-lipid membrane of the exosome could prevent fusion and thereby release of cardioprotective content to the target cell.

Having established the functional improvements of MSC-derived exosomes after myocardial I/R, we explored potential mechanisms for the therapeutic effect. First, we observed decreased tissue and systemic inflammation in exosome treated animals. It is likely that the reduced inflammation is secondary to the reduced cardiac injury after exosome treatment, since leukocyte influx did not differ between the groups at 1 hour reperfusion. Cardioprotective interventions targeting primarily inflammation have usually profound anti-inflammatory effects at very early time points after reperfusion.[14, 16]

Secondly, MSC-derived exosomes appear to activate specific pro-survival pathways. Compared to saline treatment, exosomes significantly enhanced Akt and GSK3 phosphorylation whereas phosphorylation of ERK1/2 was not altered. It is difficult to address whether exosomes phosphorylate Akt and GSK3 independent from each other. It is known that phosphorylated Akt in turn phosphorylates GSK3, thereby inactivating detrimental actions of GSK3 on cardiomyocyte mitochondria.[27] Nevertheless, in our study the net effect of phosphorylated Akt and (subsequent) GSK3 is enhanced survival of myocardial cells after I/R injury. These findings corroborate earlier reports in which the paracrine mechanisms of MSC transplantation in experimental MI are mediated by Akt phosphorylation[3-7]. Hereafter, we assessed the level of apoptotic signaling and subsequent apoptosis. Phosphorylation of c-

JNK is a known mediator of apoptosis in myocardial I/R injury.[22] MSC-derived exosomes reduced c-JNK activation, thereby, together with enhanced survival kinase phosphorylation, reducing apoptosis of myocardial cells.

Finally, we studied whether MSC-derived exosomes were also able to prevent fast occurring detrimental events in I/R injury. It is known that reperfusion-induced apoptosis is an active process causing ATP depletion within the first few minutes after reperfusion. In addition, reperfusion allows restoration of aerobic glycolysis in which energy is transferred to NADH. Subsequently, NADH is oxidized into NAD+ during oxidative phosphorylation in order to generate ATP in the mitochondria. We have shown that exosome treatment results in reduced ATP depletion and increased NADH availability compared to saline treatment. This effect was significantly present as early as 30 minutes after reperfusion. Whether exosomes increase ATP and NADH availability directly, via decreased injury to mitochondria (phosphorylated Akt/GSK3 pathway results in reduced mitochondrial damage) or promoted glycolysis remains to be addressed.

In conclusion, we have shown that MSC-derived exosomes reduce infarct size and prevent heart function deterioration after myocardial I/R injury. The main mode of action of MSC-derived exosomes is enhancing viability, preventing apoptosis of myocardial cells and restoring energy balance. More importantly, our study demonstrates that MSC-derived exosomes are effective when administered in the late ischemic period, just prior to reperfusion. For this reason, MSC-derived exosomes are a potential candidate for adjunctive therapy for patients suffering from acute myocardial infarction.

Acknowledgements

We would like to thank the following persons for their assistance: Ben J. van Middelaar and Arjan Schoneveld (all from the UMC Utrecht, The Netherlands).

Funding Sources

This work is supported by research grants from the European Community's 6th Framework Program (contract LSHMCT-2006-037400, IMMUNATH), Utrecht University Mozaïek grant (contract 017.004.004 to F.A.), Netherlands Heart Foundation (contract 2010.T001 to F.A.) and Bekales Foundation (P.A.D.)

Disclosures

None

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Table 1. Invasive left ventricular pressure measurements 28 days after infarction

	Saline	Exosomes	*p*
BPM	480±7	476±3	.60
ESP	110±2	137±10	.12
EDP	15±1	7±1	.009
dP/dT_{max}	7496±152	8951±246	.009
dP/dT_{min}	-6680±75	-8241±396	.009

Data are represented as Mean±SEM, *n*=5/group. BPM=beats per minute, ESP=end-systolic pressure, EDP=end-diastolic pressure.

Figure 1. MSC-derived exosomes reduce myocardial I/R injury *in vivo* and *ex vivo*. A, Infarct size (IS) as a percentage of the area-at-risk (AAR) 1 day after I/R injury; **p*<0.001 and †*p*=0.002 compared to saline. B, AAR as a percentage of the left ventricle (LV). Each bar represents Mean±SEM, n=6/group for *in vivo* and n=4/group for *ex vivo* experiments.

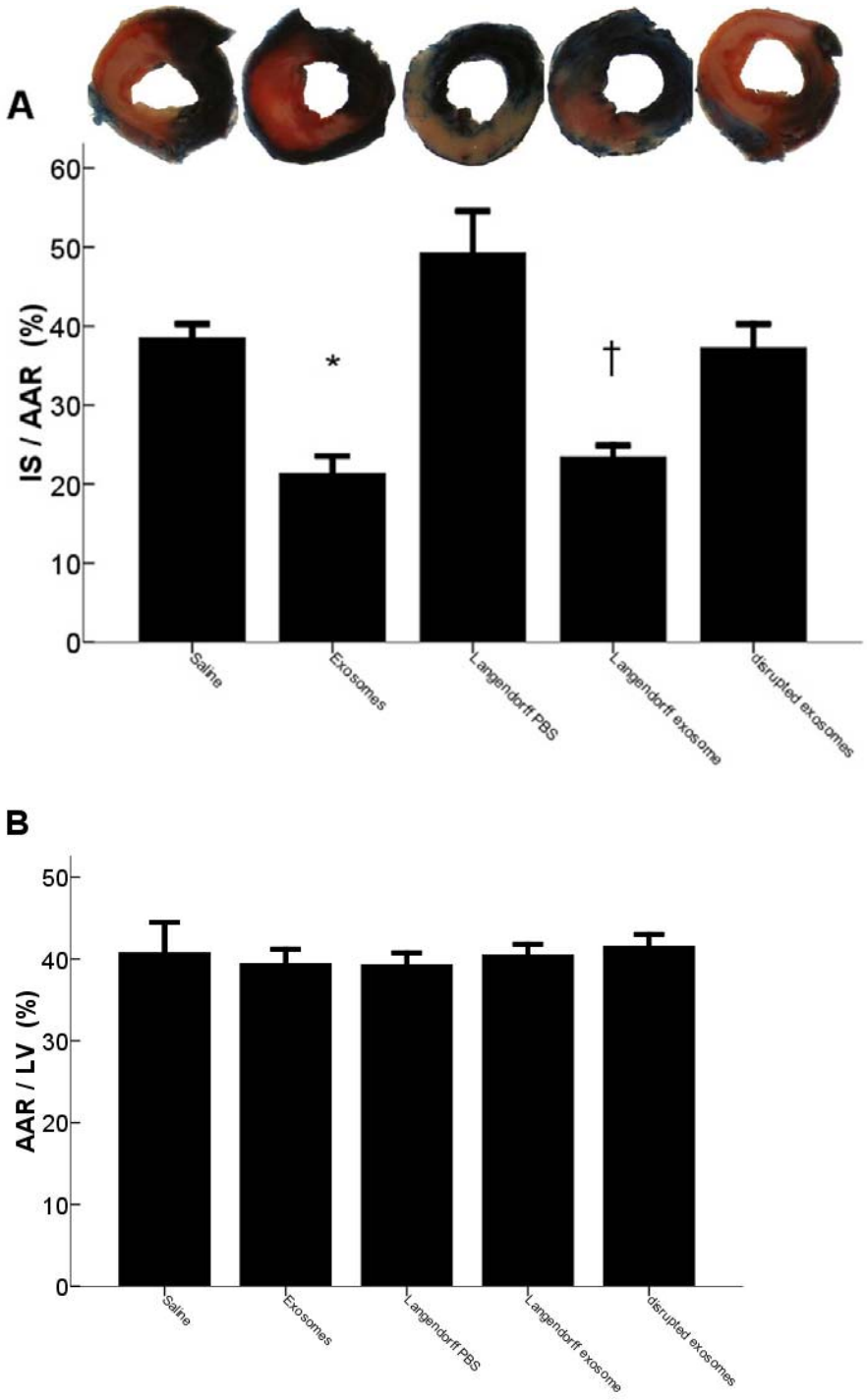
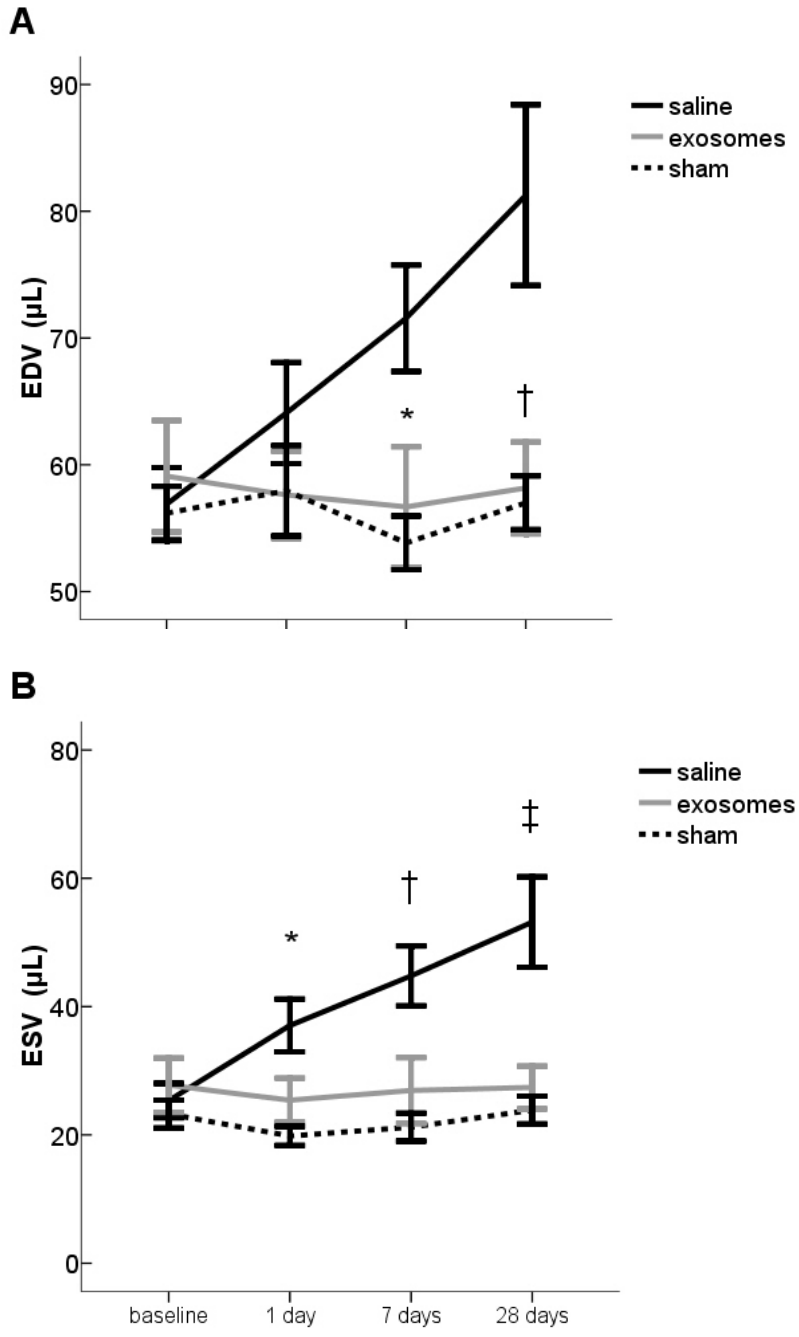
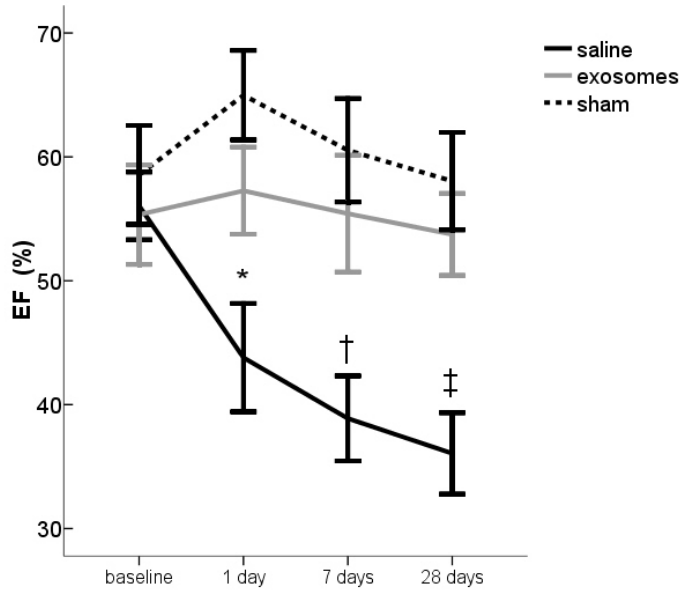


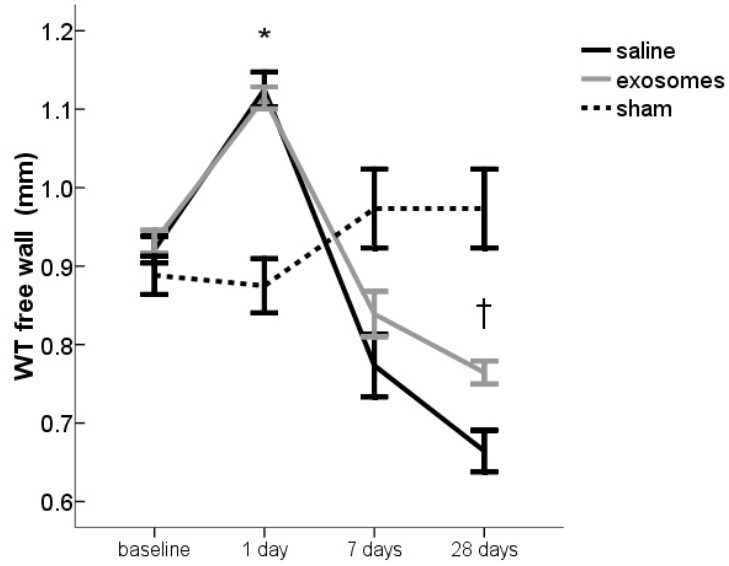
Figure 2. MSC-derived exosomes prevent LV dilation and improve systolic function after myocardial I/R injury. A**,** End-diastolic volume (EDV, µL); **p*=0.029 and †*p*=0.006 compared to saline. B, End-systolic volume (ESV); **p*=0.04, †*p*=0.017 and ‡*p*=0.002 compared to saline. C, Ejection fraction (EF, %); **p*=0.035, †*p*=0.015 and ‡*p*=0.002 compared to saline. D, Wall thickness of the infarct area (WT, mm); **p*<0.001 compared to baseline and †*p*=0.04 compared to saline. E, WT of the remote myocardium. F, systolic wall thickening (SWT, %); **p*=0.011, †*p*=0.042 and ‡*p*=0.040 compared to saline. G, SWT of the remote myocardium; **p*=0.012 compared to saline. Representative MRI images are shown. Note the increased LV dimensions at systole and diastole in saline treated animals; Each bar represents Mean±SEM, n=10/group for ischemic/reperfused animals n=6/group for sham operated mice.



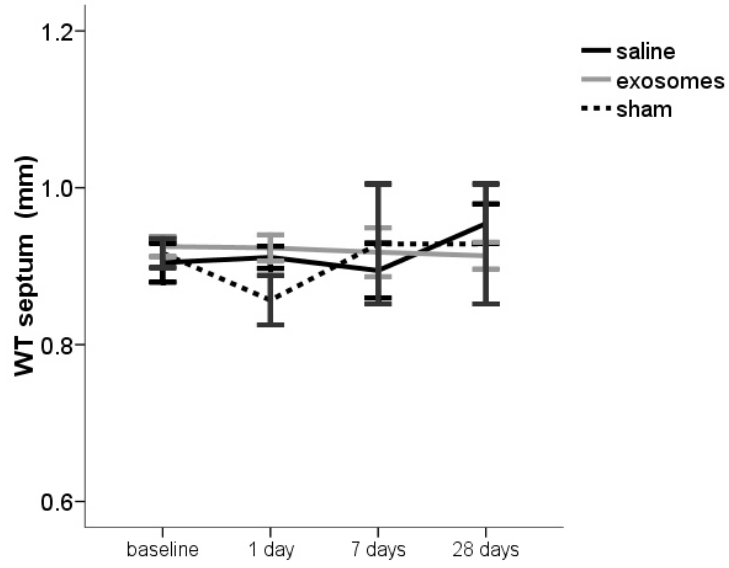
C


70
60
50
40
30
EF (%)
*
†
‡
baseline
1 day
7 days
28 days
saline
exosomes
sham

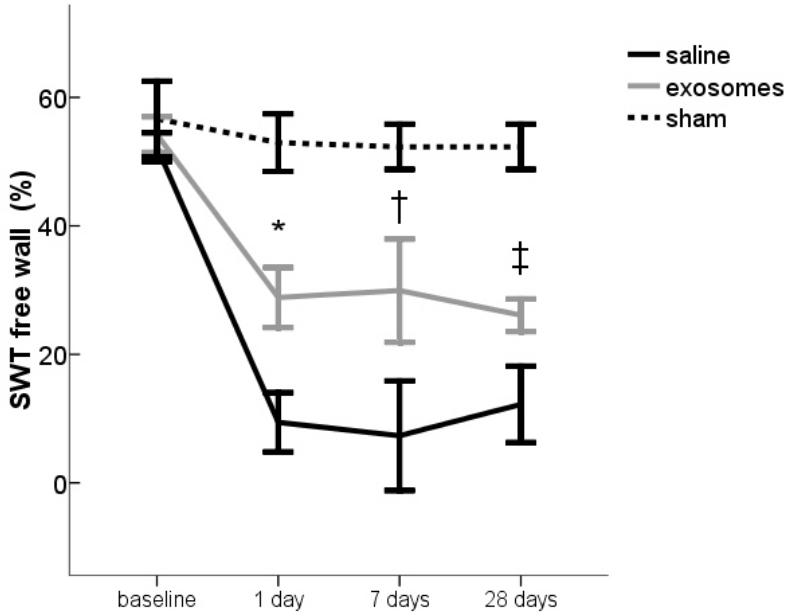
D


1.2
1.1
1.0
0.9
0.8
0.7
0.6
WT free wall (mm)
*
†
baseline
1 day
7 days
28 days
saline
exosomes
sham

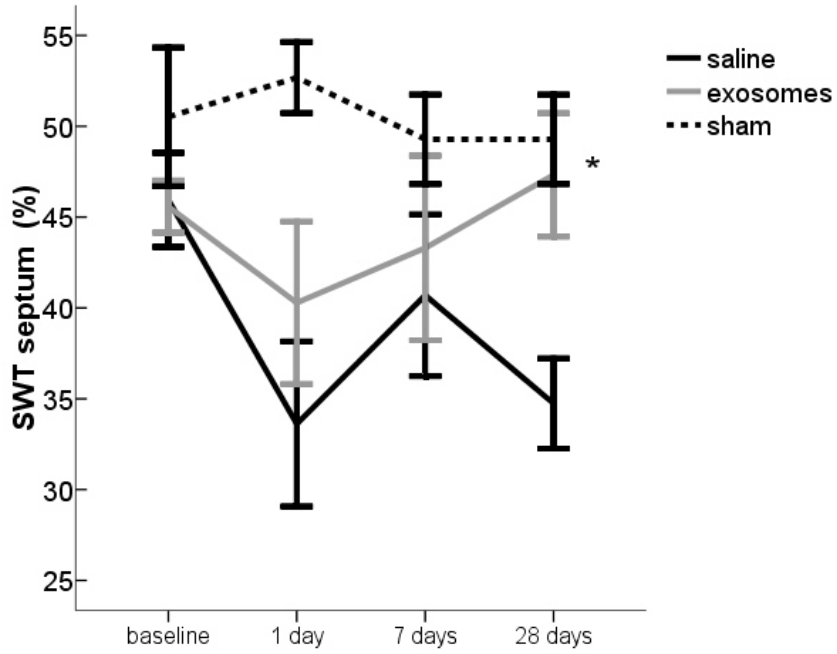
E


1.2
1.0
0.8
0.6
WT septum (mm)
baseline
1 day
7 days
28 days
saline
exosomes
sham

F


saline
exosomes
sham
SWT free wall (%)
60
40
20
0
*
†
‡
baseline
1 day
7 days
28 days

G


saline
exosomes
sham
SWT septum (%)
55
50
45
40
35
30
25
*
baseline
1 day
7 days
28 days

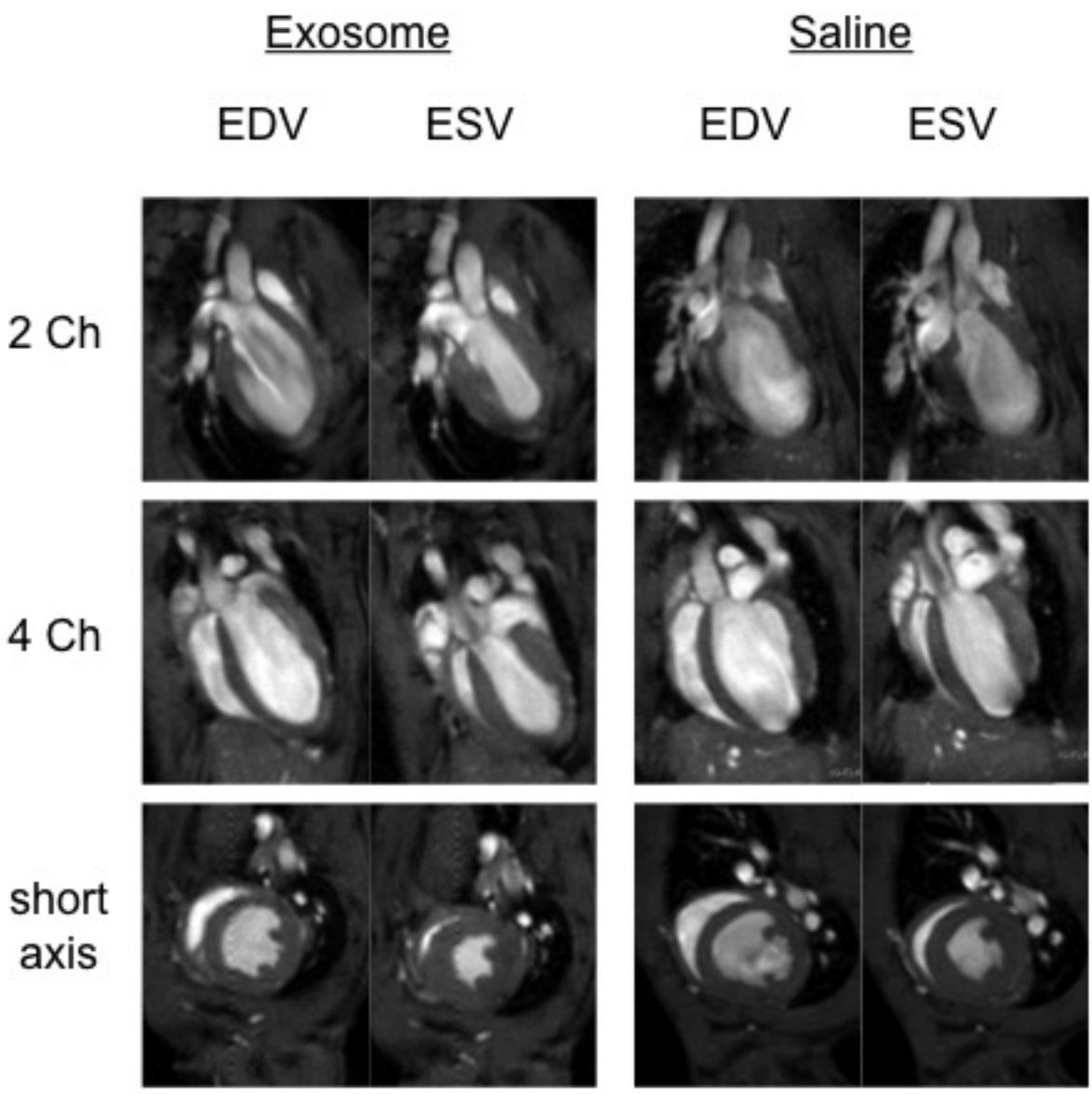
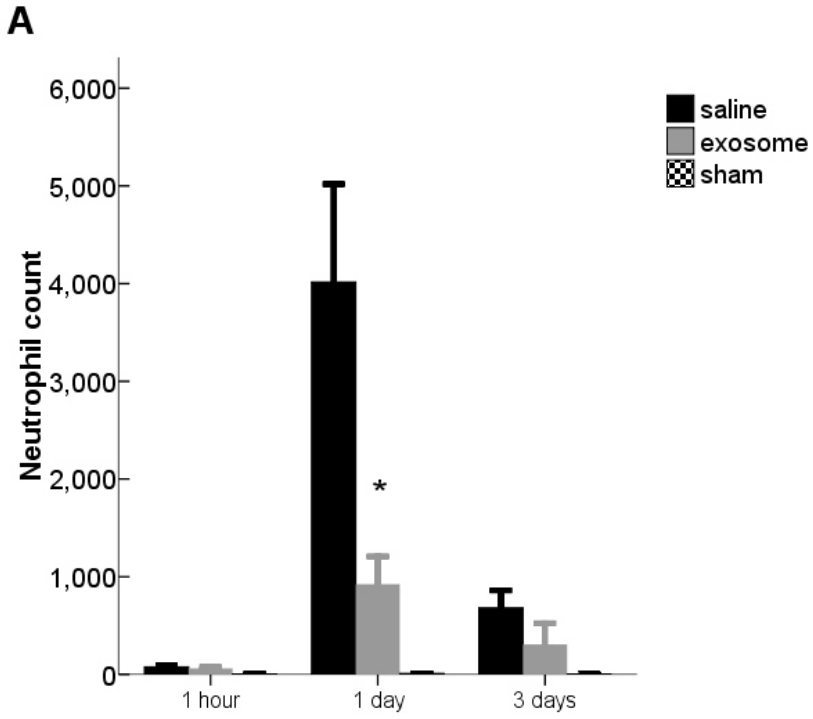
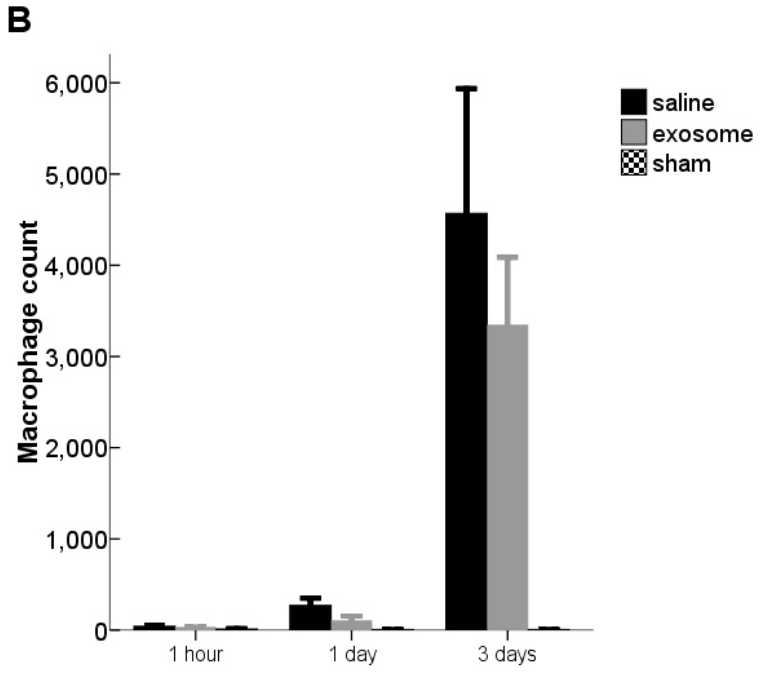
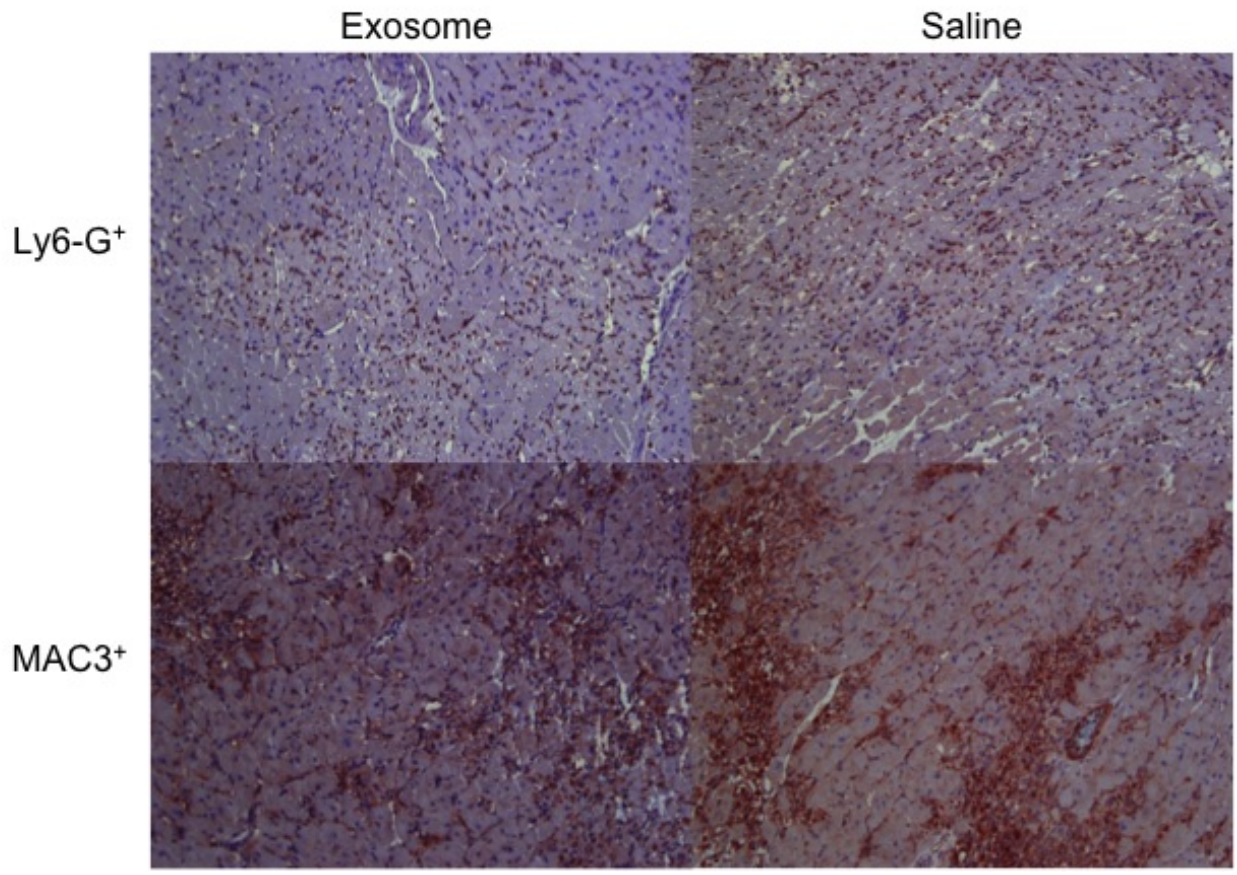

Exosome
Saline
EDV
ESV
EDV
ESV
2 Ch
4 Ch
short axis

Figure 3. MSC-derived exosomes reduce secondary inflammation after myocardial I/R injury. A**,** Neutrophil influx (Ly6-G^{pos} cells); **p*=0.016 compared to saline. B, Macrophage influx (MAC3^{pos} cells). Representative staining are shown. C, Peripheral white blood cell counts after myocardial I/R injury; **p*=0.045 and †*p*=0.009 compared to saline. Each bar represents Mean±SEM, n=6/group/time point.






Exosome
Saline
Ly6-G+
MAC3+

C

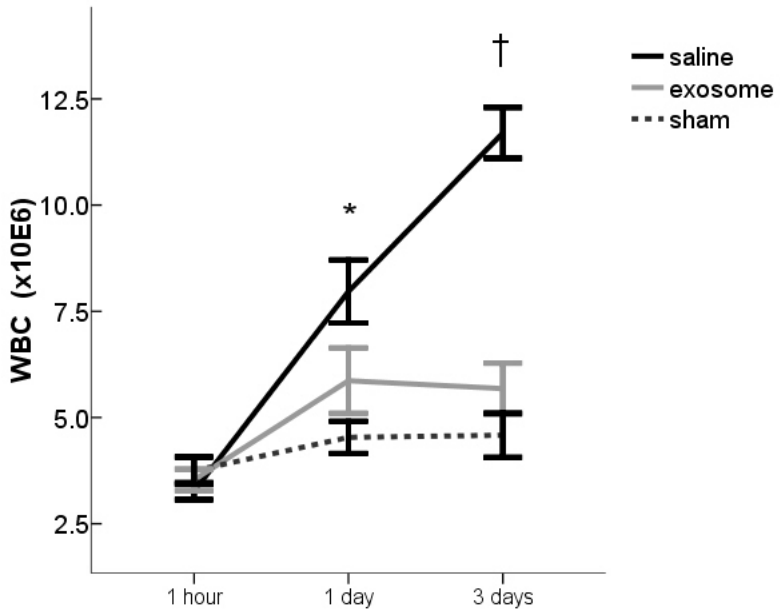
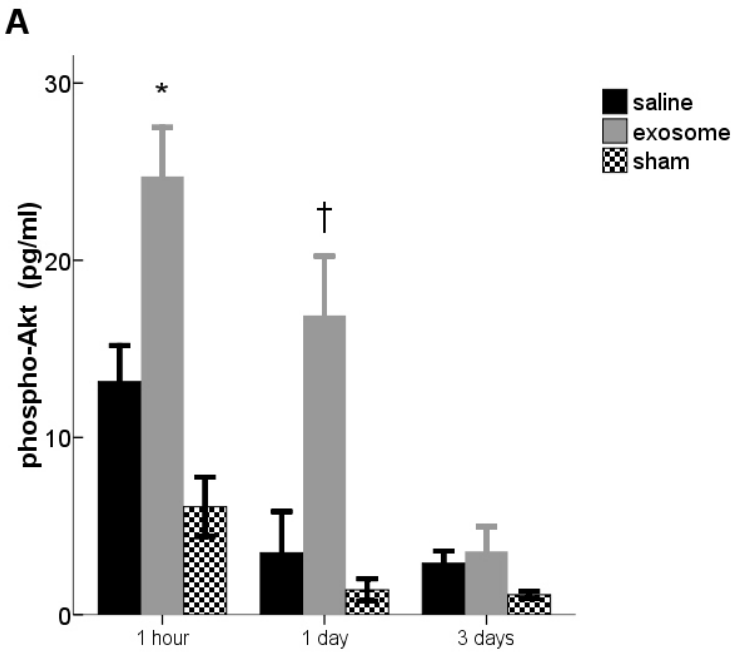
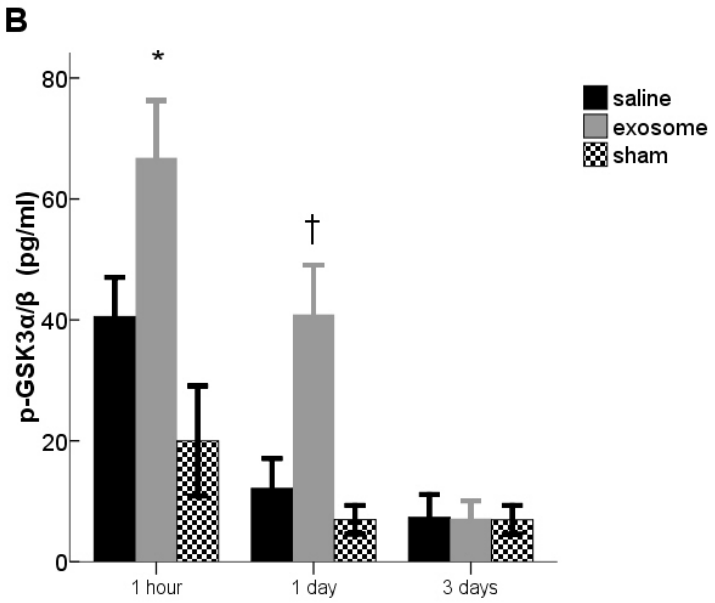

WBC (x10E6)
12.5
10.0
7.5
5.0
2.5
1 hour
1 day
3 days
*
†
saline
exosome
sham

Figure 4. MSC-derived exosomes reduce apotosis via induced phosphorylation of Akt and GSK3, and reduced c-JNK phosphorylation after myocardial I/R injury. Tissue levels of A**,** phospho-Akt (pg/ml); **p*=0.025 and †*p*=0.025 compared to saline. B, phospho-GSK3α/ß; **p*=0.025 and †*p*=0.016 compared to saline. C, phospho-ERK1/2. D, phospho-c-JNK; **p*=0.045 compared to saline. E, TUNEL^{pos} nuclei in hearts 1 day after I/R injury. Representative images are shown above the bars; **p*=0.009 compared to saline. Each bar



represents Mean±SEM, n=6/group/time point.



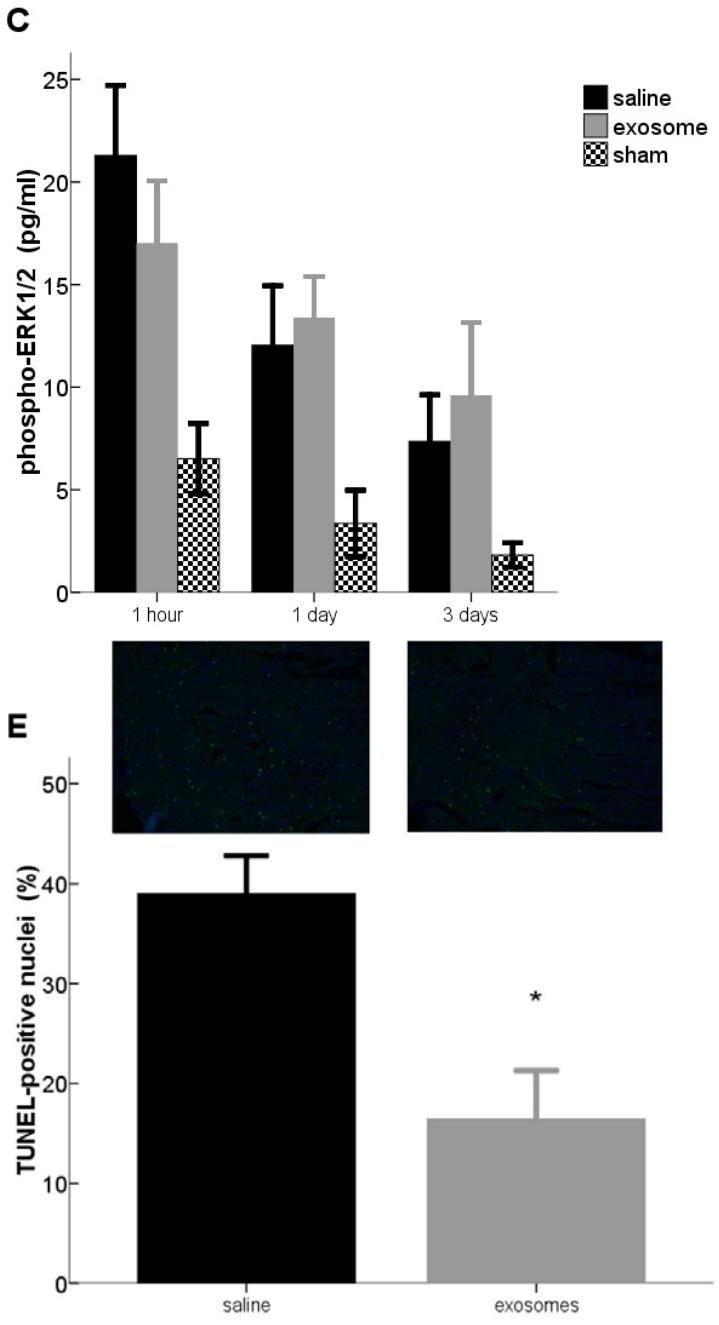
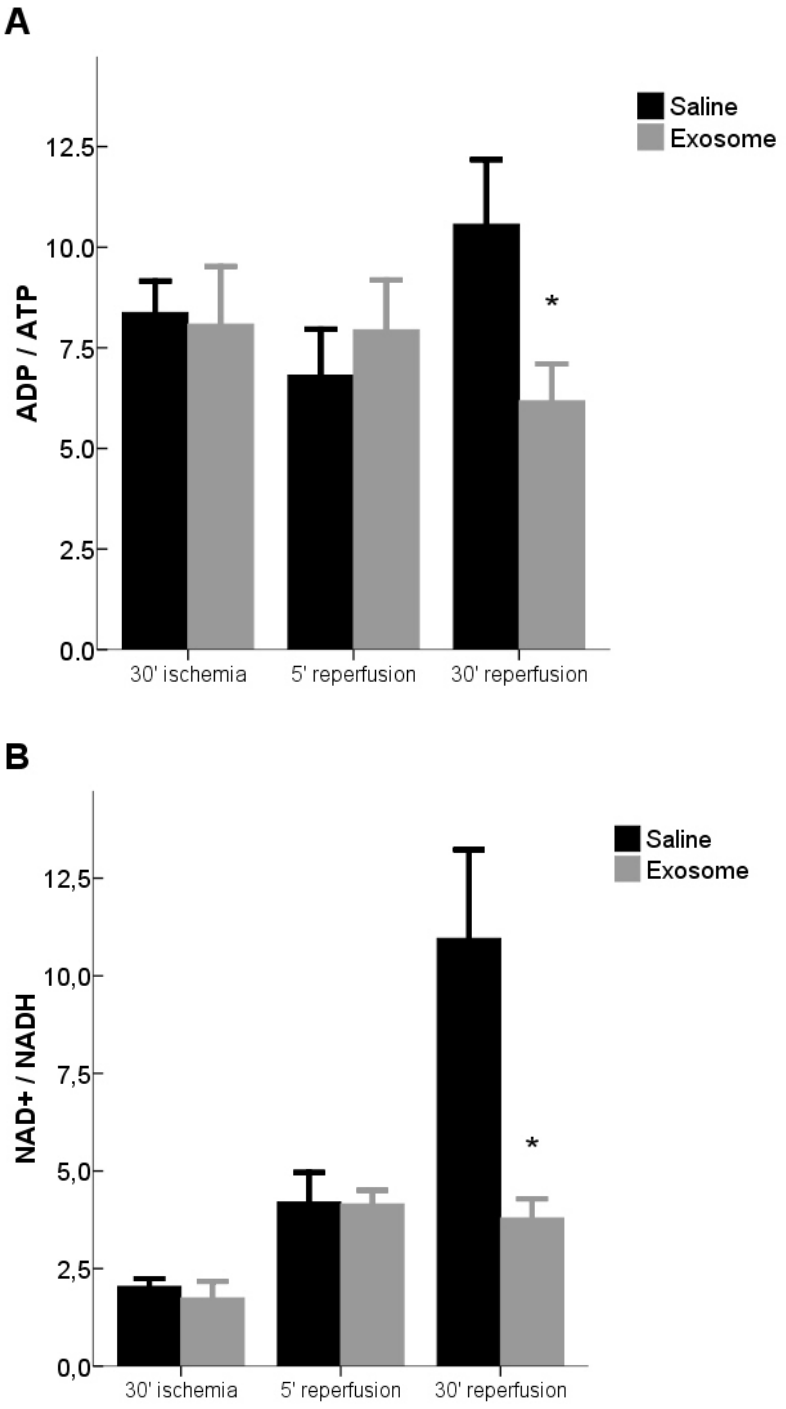

C
phospho-ERK1/2 (pg/ml)
25
20
15
10
5
0
saline
exosome
sham
1 hour
1 day
3 days
E
TUNEL-positive nuclei (%)
50
40
30
20
10
0
*
saline
exosomes

Figure 5. MSC-derived exosomes restore ADP/ATP and NAD+/NADH levels and inhibit MAC formation. Tissue levels of A**,** ADP/ATP; **p*=0.047 compared to saline. B, NAD+/NADH; **p*=0.027 compared to saline. C, MACs; **p*=0.045 compared to saline. Each bar represents Mean±SEM, n=8/group/time point.



Supplemental Material

Exosome Purification

Exosomes were purified from huES9.E1 derived MSCs conditioned media (CM) using HPLC as described earlier.[1] 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.

Infarct Size

Infarct size (IS) as a percentage of the left ventricle (LV) was determined using Evans' blue dye injection and TTC staining, 1 day after reperfusion (n=6/group). The LCA was ligated once again at the level marked by the suture left in place. Evans' blue dye (4%) was injected via the thoracic aorta in a retrograde fashion. By doing so, one can demarcate the area-at-risk (AAR), the extent of myocardial tissue that underwent ischemia (i.e. endangered myocardium). Hearts were rapidly explanted, rinsed in 0.9% saline and put in -20ºC freezer for 1 hour. Hereafter, hearts were mechanically sliced into four 1-mm cross sections. Heart sections were incubated in 1% triphenyltetrazolium-chloride (Sigma-Aldrich) at 37ºC for 15 minutes before placing them in formaldehyde for another 15 minutes. Viable tissue stains red and infarcted tissue appears white. Heart sections were digitally photographed (Canon EOS 400D) under a microscope (Carl Zeiss®). IS, AAR

and total LV area were measured using ImageJ software (version 1.34). Infarct size was corrected for the weight of the corresponding heart slice.

Myocardial I/R Injury *Ex Vivo*

Mice (n=4/group) were given heparin 50 IE subcutaneously. The suture was placed beneath the LCA *in vivo* without ligating. Hereafter, the heart was excised and aortic root was canulated and perfused in the Langendorff setup. After 10 min recovery, the suture was tightened to induce ischemia for 30 min. Just 5 min prior to reperfusion, the perfusion buffer was changed for a second buffer containing 0.4 µg/ml MSC-derived exosomes or control. Reperfusion was initiated by releasing the suture. Following 3 hours of reperfusion, Evans' blue dye was injected after re-ligating the suture to demarcate the AAR. Subsequently, TTC staining was performed for infarct size assessment.

Cardiac Magnetic Resonance Imaging

Twenty-six mice (*n*=10/group in ischemic and n=6/group in sham operated mice) underwent serial assessment of cardiac dimensions and function by high resolution magnetic resonance imaging (MRI, 9.4 T, Bruker, Rheinstetten, Germany) under isoflurane anesthesia before, 1, 7 and 28 days after MI. Long axis and short axis images with 1.0 mm interval between the slices were obtained and used to compute end-diastolic volume (EDV, largest volume) and end-systolic volume (ESV, smallest volume). The ejection fraction (EF) was calculated as 100*(EDV-ESV)/EDV. Wall thickness (WT) and systolic wall thickening (SWT) were assessed from both the septum (remote myocardium) and free wall (infarct

area). All MRI data are analyzed using Qmass digital imaging software (Medis, Leiden, The Netherlands).

Pressure-Volume Loop Recordings

In a subset of mice, invasive assessment of cardiac performance and LV pressure development was performed 28 days after infarction. A Millar 1.4F pressure catheter (model SPR-839) was inserted in a retrograde fashion via the right common carotid artery. Systolic function was assessed by LV end-systolic pressure and dP/dt_{max}, whereas diastolic function by LV end-diastolic pressure and dP/dt_{min}.

Immunohistochemistry

Upon termination, hearts were excised and fixated in 4% formaldehyde and embedded in paraffin. Paraffin sections were stained for Ly-6G (for neutrophils; rat anti-mouse Ly-6G 1:100, Abcam, Cambridge, United Kingdom), MAC-3 (for macrophages; rat anti-mouse MAC-3 1:30, BD Pharmingen, Breda, the Netherlands) and Terminal deoxynucleotidyl transferase dUTP Nick End labeling (TUNEL) staining.

Sections were stained by overnight incubation with the first antibody at 4°C for MAC-3 or by 1 hour incubation at RT for Ly-6G. Before staining, sections were deparaffinized and endogenous peroxidase was blocked by 30 minutes incubation in methanol containing 1.5% H2O2. Antigen retrieval was performed by 20 minutes boiling in citrate buffer (MAC-3 and Ly-6G).

For MAC-3, sections were pre-incubated with normal goat serum and incubated with the primary antibody (1:30 overnight at 4°C). Sections were then incubated for 1 hour at RT with a biotin labeled secondary antibody, followed by 1 hour incubation with streptavidin-horseradish peroxidase at RT and developed with AEC.

For Ly-6G, sections were incubated with the primary antibody (1:100 for 1 hour at RT). Sections were then incubated for 30 minutes with a secondary antibody followed by 30 minutes incubation with Powervision poly-HRP anti-rabbit IgG (ImmunoVision Technologies, Daily City, USA). The staining was immediately visualized with Vector NovaRED^{TM} substrate kit following the manufacturer's instructions (Vector Laboratories Inc., Burlingame, USA).

Protein Isolation

Total protein was isolated from snap frozen infarcted heart sections using 1 ml Tripure^{TM} Isolation Reagent (Roche) according to the manufacturers' protocol.

Flow Cytometry

Phosphorylated target protein for Akt (Ser^{473}), glucogen synthase kinase-3α/β (GSK-3α/β; Ser^{21}/Ser^{9}), extracellular signal-regulated kinases-1/2 (ERK1/2; Thr^{202}/Tyr^{204}), c-Jun N-terminal kinase (c-JNK; Thr^{183}/Tyr^{185}) were measured using the Bio-Plex Multiplex Assay (Bio-Rad Laboratories) according to the instructions of the manufacturer, after 1:5 dilution in assay buffer.

ADP/ATP and NAD+/NADH measurementADP/ATP and NAD+/NADH ratio in area at risk of mouse model MI/R injury were measured. MI was induced by 30

min left coronary artery (LCA) occlusion and subsequent reperfusion. Five minutes before reperfusion, mice were intravenously infused with 200 µl saline diluted exosomes containing 0.4 µg protein via the tail vein. Control animals were infused with 200 µl saline. After 0 min, 5 min and 30 min of reperfusion, area at risk (AAR) was excised and frozen immediately using liquid nitrogen. Frozen tissue samples were then homogenized with 1 ml of NAD+/NADH extraction buffer (Biovision, Mountain view, CA) and supernatant was collected after spinning at 14,000 xg for 5 minutes. After that, the supernatant was filtering through 10 kDa MWCO filter (Biovision) before performing the ADP/ATP and NAD+/NADH ratio assays according to the assay manual (Biovision). For ADP/ATP and NAD+/NADH ratio assay, 5 µl or 3 µl of supernatant was used respectively.

1. Lai RC, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, Salto-Tellez M, Timmers L, Lee CN, El Oakley RM, Pasterkamp G, de Kleijn DP, Lim SK. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res.* 2010;4(3):214-222.

CONCLUSION

This conclusion are adapted with modifications from a published review article "Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease"[1] of which I am the first author.

Exosomes As The Cardioprotective Component

The discovery that MSC secretion reduced myocardial infarct size after ischemia/reperfusion[71,72] sparks the possibility of identifying a secreted cardioprotective factor to reduce the infarct size of AMI patients undergoing reperfusion therapy. A secreted factor would be potentially much simpler to produce or manufacture, to administer to patient and to understand.

This thesis accomplished this by identifying exosome, a bi-lipid membrane vesicle containing proteins and RNAs, as the cardioprotective factor in MSC secretion. By reducing the complexity of MSC secretion to a single entity, amelioration of reperfusion injury during reperfusion therapy of myocardial ischemia could be achieved through exosomes instead of MSC transplantation. This would circumvent the safety concerns and limitations associated with cell transplantation. These include the proarrhythmic effects when the donor cells fail to couple with the host tissue[81-83]; the risk that the biological potency of the transplanted cells may persist even if treatment is terminated as a result of adverse outcomes; the long-term safety concerns of the ossification and/or calcification in tissues due to the potential of MSCs to differentiate into osteocytes and chondrocytes[84]; the risk of occlusion of intravascularly administered MSCs in the distal microvasculature

due to the relatively large size of MSCs[85] and other limitations that are generally universal in cell-based therapy such as the immediate availability of large quantity of cells and the costly storage and administration of viable cells.

Besides mitigating the risks associated with cell transplantation, exosomes can also circumvent some of the challenges associated with the use of small soluble biological factors such as growth factors, chemokines, cytokines, transcription factors, genes and RNAs[77]. The delivery of soluble factors to the right cell type and in the case of those factors that work intracellularly, delivery into the right cellular compartments while maintaining the stability, integrity and biological potency of these factors during manufacture, storage and subsequent administration remain challenging[77]. As a bi-lipid membrane vesicle, exosomes not only have the capacity to carry a large cargo load, but also protect the contents from degradative enzymes or chemicals. For example, protein and RNA in MSC exosomes were protected from degradation by trypsin and RNase as long as the lipid membrane was not compromised[86,87]. We also found that storage without potentially toxic cryopreservatives at -20°C for 6 months did not compromise the cardioprotective effects of MSC exosomes or their biochemical activities (Lai RC, Lim SK, unpublished data). The bi-lipid membrane, also allows exosomes to circumvent many of the challenges associated with small soluble biological factors including the maintenance of stability, integrity and biological potency during manufacturing, storage and administration[77].

Exosomes As The Therapeutic Agent

In our profiling of the exosome proteome, we identified more than 800 proteins and uncovered a diverse range of biochemical and cellular potential of MSC exosomes. These observations suggested that despite being smaller than a cell, exosomes are relatively complex biological entities making them ideal therapeutic candidates to treat complex injuries such as MI/R injury[7] that occurs during treatment to restore oxygen to ischemic heart tissues of AMI patients. During reperfusion, the restoration of blood and oxygen to ischemic myocardium paradoxically exacerbates the ischemia-induced cellular insults[7]. This is because the biochemical cascades required for cell survival that are initiated by cells during no-flow ischemia[88] are not compatible with the rapid restoration of flow and oxygen supply, and at the same time, the ischemic cells cannot alter their biochemical activities expeditiously enough to adapt to this restoration. This latter phenomenon was best evidenced by the reduction of MI/R injury through postconditioning where cells were exposed to repeated short nonlethal cycles of reperfusion/ischemia to facilitate biochemical adaptation to reperfusion[89-100].

We postulate that with their complex cargo, exosomes would have adequate potential to participate in a wide spectrum of biochemical and cellular activities, and mitigate some of the MI/R injuries such as ATP deficit due to mitochondrial damage[101], activation of complements[102,103], oxidative stress[7] and cell death. We demonstrated that exosomes could enhance glycolysis and thereby circumvent mitochondrial damages to increase ATP production. Exosomes could also ameliorate complement activation by inhibiting the formation of membrane attack complex through CD59 on their membranes. The presence of intact functional 20S proteasome in exosomes could reduce oxidative stress by degrading oxidized

proteins. Exosomes could enhance cell survival by activating adenosine-mediated signaling pathway to phosphorylate ERK1/2 and AKT through adenosine produced by CD73 on their membrane. Furthermore, exosome treatment in a mouse model MI/R injury activated pro-survival kinases by phosphorylating AKT[104], GSK3[105], inhibited mediator of apoptosis by dephosphorylating c-JNK[106], and at the same time increased ATP production, enhanced redox balance possibly through glycolysis within the 1st hour of reperfusion. These observations demonstrated the biochemical potential of exosomes and suggested possible mechanisms for the reduction of MI/R injury. Furthermore, the results that exosomes reduced infarct size in the Langendorff setup of the I/R experiment suggested exosomes might promote these cardioprotective mechanisms directly in myocardial cells.

In addition to its potential to target many processes simultaneously, a hallmark feature of exosome is the exertion of its effects through enzymes. Since enzyme activities are catalytic rather than stoichiometric, and are dictated by their microenvironment (e.g., substrate concentration or pH), the enzyme-based therapeutic activities of exosomes could be activated or attenuated according to the release of injury-associated substrates, which in turn, is proportional to the severity of disease-precipitating microenvironment. Resolution of the disease-precipitating microenvironment would reduce the release of injury-associated substrates and also the activity of exosome enzymes. Consequently, the efficacy of exosome-based therapeutics could be highly responsive to, but also limited by, the disease-precipitating microenvironment.

Exosomes are known to bear numerous membrane proteins that have binding affinity to other ligands on cell membranes or the extracellular matrix, such as

transferrin receptor, tumor necrosis factor, lactadherin, integrins and tetraspanin proteins (e.g., CD9, CD63 and CD81)[107]. These membrane bound molecules provide a potential mechanism for the homing of exosomes to a specific tissue or microenvironment. For example, integrins on exosomes could home exosomes to cardiomyocytes that express ICAM1, a ligand of integrins after MI/R injury[108], or to VCAM-1 on endothelial cells[109]. Besides, tetraspanin proteins, which function primarily to mediate cellular penetration, invasion and fusion events[110], could facilitate cellular uptake of exosomes by specific cell types.

Together, the features discussed here render exosome a highly efficacious therapeutic candidate in neutralizing the complexity of MI/R and a potential adjuvant to complement current reperfusion therapy. Furthermore, during 28 days follow up in the MI/R injury mouse model, we observed that exosome treatment which significantly reduced infarct size after reperfusion also significantly preserved both cardiac function and geometry. This is consistent with the findings that a smaller infarct size prevents heart function deterioration and retards the progress to heart failure [8-13].

More importantly, the animal models used in the demonstration of MSC exosome-mediated cardioprotection had two important features that highlighted the clinical relevance and practicality of an exosome-based adjuvant to reperfusion therapy. Both animal models used in our study had experimentally induced lethal ischemia that causes similar level of pathological substrate as that in the average patient with AMI[72]. Exosome was administered to these animals just before reperfusion, which is a clinically feasible time point. These two features facilitate the translation of MSC exosome into an adjunctive therapy to reduce reperfusion injury during treatment of AMI patients .

Exosome As MSCs' Vehicle of Choice for Intercellular Communication

Besides the involvement of exosomes in MI/R injury, this thesis also showed that production of cardioprotective exosomes was not restricted to hESC-MSCs as different fetal tissue-MSCs were also found to produce cardioprotective exosomes. This suggested that producing protective exosomes might be a universal property of all MSCs. This property may be a reflection of the stromal support role of MSCs in maintaining a microenvironmental niche for other cells such as hematopoietic stem cells[111,112]. The secretion of exosomes may also be a dominant function of MSCs. We recently observed that when GFP-labeled exosome-associated protein CD81, a tetraspanin membrane protein usually found localized to the plasma membrane, is expressed in hESC-MSCs, they exhibit a punctate cytosolic distribution and these labeled proteins were secreted (see Figure 2 of review attached in appendix)[1]. The difference in cellular distribution and secretion of CD81 in hESC-MSCs and HEK293 cells suggest that MSCs are prolific producers of exosomes. This leads to the speculation that exosome might be the MSCs' vehicle of choice for intercellular communication.

Future Challenge

The future challenge of this study is to translate this finding into a clinically useful therapeutic agent. The first major challenge would be to manufacture Good Manufacturing Practice (GMP) grade exosomes from non-autologous cell sources. Although exosomes have already been tested in clinic as a form of cancer vaccine[113-115], exosomes used for these tests were produced during short-term *ex*

vivo culture of autologous dendritic cells. For this reason, manufacturing of these exosomes cannot provide sufficient guidance for the MSC exosomes GMP production. This manufacturing process faces many unique challenges, which involve ethical, legal, technical, monetary and regulatory/safety issues.

The other major challenge will be the cell source for derivation of MSCs since the use of hESCs for the derivation of MSCs presents both ethical and legal challenges. While ethical objections to the derivation and use of hESCs have initially hindered hESC research, they have abated. Instead, the use and applications of hESCs is now being hindered by complex and widespread patenting in some countries[116] and the ban on stem cell-related patents in other countries[117]. To encourage the development of hESC-based therapeutic applications, the need for freedom to use and share hESC resources and knowledge must be balanced with a need to incentivize commercial development of stem cells by protecting the intellectual property generated from research and development efforts. Unfortunately, this balance has not yet been reached.

Another technical challenge is the finite expansion capacity of hESC-MSC. This resulted in the need for constant re-derivation of MSCs and re-validation of each of the derived cell batch, which are both time consuming and costly. Therefore, a robust scalable and highly renewable cell source will be central to the development of a commercially viable manufacturing process for the production of MSC exosomes in sufficient quantity and quality to support clinical testing or applications. To address this issue, we demonstrated that immortalization of the hESC-MSCs by Myc did not compromise the quality or yield of exosomes[118]. Therefore, this provides a potentially inexhaustible cell source for MSC exosome production. The translation of MSC exosomes into clinical applications is also

complicated by the relative novelty of exosomes with few precedents in the regulatory and safety space of biopharmaceuticals. This will require the formulation of new standards for manufacture, safety and quality control.

With the demonstration of the involvement of exosomes in cardioprotection and the proteomic prediction of its therapeutic potential, we speculate that these exosomes will have similar positive effect in other reperfusion injuries such as those involved in stroke and renal ischemia disease. On the other hand, it is also possible that different cell types produce exosomes that are specific for certain type of cells or injuries. If true, this novel tissue-repair function of exosomes could potentially engender new approaches to the development of biologics that are much needed in the clinic.

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APPENDICES

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Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease

Cardiovascular disease is a major target for many experimental stem cell-based therapies and mesenchymal stem cells (MSCs) are widely used in these therapies. Transplantation of MSCs to treat cardiac disease has always been predicated on the hypothesis that these cells would engraft, differentiate and replace damaged cardiac tissues. However, experimental or clinical observations so far have failed to demonstrate a therapeutically relevant level of transplanted MSC engraftment or differentiation. Instead, they indicate that transplanted MSCs secrete factors to reduce tissue injury and/or enhance tissue repair. Here we review the evidences supporting this hypothesis including the recent identification of exosome as a therapeutic agent in MSC secretion. In particular, we will discuss the potential and practicality of using this relatively novel entity as a therapeutic modality for the treatment of cardiac disease, particularly acute myocardial infarction.

KEYWORDS: acute myocardial infarction ■ exosome ■ mesenchymal stem cell ■ paracrine secretion

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Stem cells in the treatment of acute myocardial infarction

Acute myocardial infarction (AMI) is the primary cause of disease-related death in the world [1–3]. It is characterized by the disruption of blood supply to the heart muscle cells, which lead to myocardial infarction or death of cardiomyocytes. Reperfusion therapy or the restoration of blood flow by thrombolytic therapy, bypass surgery or percutaneous coronary intervention (PCI) is currently the mainstay of treatment for AMI and is responsible for the significant reduction in AMI mortality [4]. The efficacy of reperfusion therapy has led to increasing survival of patients with severe AMI who would not otherwise survive. However, many (23%) of these survivors progress to fatal heart failure within 30 days [5]. This phenomenon of an increasing number of severe AMI survivors contributes to an ever growing epidemic of heart failures [6–8].

Heart failure is characterized by dilatation and hypertrophy with fibrosis within the myocardium. The progression of an AMI survivor to heart failure is a multifactorial process that has been hypothesized to include the development of myocardial stunning and hibernation, remodeling and chronic neuroendocrine activation [9], and is dependent on the extent of the AMI suffered by the patient [10–15]. The development of reperfusion therapy and its subsequent improvements have significantly increased the salvage of ischemic myocardium from infarction and reduced infarct size, but further substantive improvement to reperfusion therapy is likely to require adjunctive therapies.

Although it was recognized as early as 1960 that reperfusion of severely ischemic tissue causes lethal injury [16], the concept that reperfusion causes *de novo* lethal injury became more widely accepted only when infarct size was shown to be reduced by interventions applied at the onset of reperfusion (reviewed in [10]). Such interventions, also known as postconditioning, involve ischemic conditioning or application of pharmacological agents before the onset of reperfusion, and have demonstrated some protection against reperfusion injury in animals and in small clinical trials. However, none of these agents have proven to be efficacious in large clinical trials and this has led to speculations that reducing reperfusion injury may not be tractable to pharmaceutical interventions [17].

With the emergence of stem cells as potential therapeutic agents, attempts to use stem cells to reduce infarct size and enhance cardiac function in animal models and patients have increased exponentially. To date, stem cell therapy for the heart accounts for a third of publications in the regenerative medicine field [18]. Mummery *et al.* have recently reviewed the use of both adult and embryonic stem cells, such as bone marrow-derived stem cells, which include hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs), endogenous cardiac progenitor cells (CPCs), human embryonic stem cells (hESCs),

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induced pluripotent stem cells, and hESC-derived cardiomyocytes [18]. The use of bone marrow-derived stem cells such as HSCs and MSCs to repair cardiac tissues was predicated on the hypothesis that these cells could differentiate into cardiomyocytes and supporting cell types. However, careful rodent experimentation has demonstrated that few of the transplanted bone marrow cells engraft and survive, and fewer cells differentiate into cardiomyocytes or supporting cells [18]. In spite of this, transplantation of bone marrow stem cells improves some cardiac functions in animal models and patients, and this has been attributed to a paracrine effect [18]. Although the presence of CPCs in fetal hearts is well established, the presence of CPCs in postnatal or adult heart remains controversial, and the possibility that the so-called CPCs from postnatal hearts are bone marrow cells has remained unresolved. Transplanted cardiomyocytes isolated from *in vitro* differentiation of hESCs and induced pluripotent stem cells could engraft in the heart to form a synctium with each other, but not with the recipient heart. This failure to couple with the recipient cardiomyocytes could cause arrhythmia, a potentially fatal condition.

Despite our still evolving understanding of stem cell transplantation in treating cardiac disease, stem cell transplantation has already being tested in clinical trials. In a recent review of more than 20 clinical trials that primarily used adult stem cells, such as bone marrow stem cells, mobilized peripheral blood stem cells and skeletal myoblasts to treat heart disease [19], the trends favored such transplantations to treat cardiac disease when measured using clinical end points of death, recurrence of AMI or hospitalization for heart failure. The failure to elicit a more robust therapeutic response has been attributed to low engraftment of cells and poor survival of engrafted cells with an untested caveat that improved engraftment and survival will enhance the therapeutical efficacy. A general consensus from these clinical trials is that bone marrow- or blood-derived stem cells do not replenish lost cardiomyocytes or vascular cells to any meaningful extent. Instead, circumstantial evidence suggests that these stem cells secrete factors that exert a paracrine effect on the heart tissues [19].

MSCs & the treatment of cardiovascular disease

Among the stem cells currently being tested in clinical trials for the heart, MSCs are the most widely used stem cells. Part of the reason for this is their easy availability in accessible tissues, such as bone marrow aspirate and fat tissue [20], and their large capacity for *ex vivo* expansion [21]. MSCs are also known to have immunosuppressive properties [22] and, therefore, could be used in allogeneic transplantation. They are also reported to have highly plastic differentiation potential that included not only adipogenesis, osteogenesis and chondrogenesis [23–28], but also endothelial, cardiovascular [29], neurogenic [30–32] and neovascular differentiation [33–35]. MSCs transplantation in most animal models of AMI generally resulted in reduced infarct size, improved left ventricular ejection fraction, increased vascular density and myocardial perfusion [36–40]. In a recent Phase I, randomized, double-blind, placebo-controlled dose-escalation clinical trial, single infusion of allogeneic MSCs in patients with AMI was documented to be safe with some provisional indications that the MSC infusion improved outcomes with regard to cardiac arrhythmias, pulmonary function, left ventricular function and symptomatic global assessment [41].

Despite numerous studies on the transplantation of MSCs in patients and animal models, insight into the mechanistic issues underlying the effect of MSC transplantation remains vague. An often cited hypothesis is that transplanted MSCs differentiate into cardiomyocytes and supporting cell types to repair cardiac tissues. However, contrary to this differentiation hypothesis, most transplanted MSCs are entrapped in the lungs and the capillary beds of tissues other than the heart [42,43]. Furthermore, depending on the method of infusion, 6% or less of the transplanted MSCs persist in the heart 2 weeks after transplantation [44]. In addition, transplanted MSCs were observed to differentiate inefficiently into cardiomyocytes [45] while ventricular function was rapidly restored less than 72 h after transplantation [46]. All these observations are physically and temporally incompatible with the differentiation hypothesis and have thus prompted an alternative hypothesis that the transplanted MSCs mediate their therapeutic effect through secretion of paracrine factors that promote survival and tissue repair [47].

Paracrine secretion of MSCs

Paracrine secretion of MSCs was reported more than 15 years ago when Haynesworth *et al.* [48] reported that MSCs synthesize and secrete a broad spectrum of growth factors and cytokines such as VEGF, FGF, MCP-1, HGF, IGF-I, SDF-1 and thrombopoietin [49–53], which exert effects on cells in their vicinity. These factors have been postulated to promote arteriogenesis [51]; support the

stem cell crypt in the intestine [54]; protect against ischemic renal [49,50] and limb tissue injury [52]; support and maintain hematopoiesis [53]; and support the formation of megakaryocytes and proplatelets [55]. Many of these factors have also been demonstrated to exert beneficial effects on the heart, including neovascularization [56], attenuation of ventricular wall thinning [39] and increased angiogenesis [57,58].

In 2006, Gnecchi *et al.* demonstrated that intramyocardial injection of culture medium conditioned by MSCs overexpressing the Akt gene (Akt-MSCs) or Akt-MSCs reduced infarct size in a rodent model of AMI to the same extent [46]. This provided the first direct evidence that cellular secretion could be cardioprotective [46,59]. The authors subsequently attributed the cardioprotective effect of the conditioned medium to the culturing of the cells under hypoxia and the overexpression of *AKT*, which induced secretion of Sfrp2. siRNA mediated-silencing of *Sfrp2* expression in Akt-MSCs abrogated the cytoprotective effect of their secretion [60].

Our group recently demonstrated that culture medium conditioned by human ESC-derived MSCs (hESC-MSCs) significantly reduced infarct size by approximately 50% in a pig and mouse model of myocardial ischemia/reperfusion (MI/R) injury when administered intravenously in a single bolus just before reperfusion [61]. However, these MSCs were derived from hESCs instead of rat bone marrow and were not genetically modified to overexpress Akt. The conditioned medium was prepared using a chemically defined medium without hypoxia treatment.

We further demonstrated through size fractionation studies that the active component was a large complex 50–200 nm in size. Using electron microscopy, ultracentrifugation studies, mass spectrometry and biochemical assays, we identified this complex as an exosome, a secreted bi-lipid membrane vesicle of endosomal origin (FIGURE 1). When purified by size exclusion using high-performance liquid chromatography, hESC-MSC exosomes also reduced infarct size, but at a tenth of the protein dosage used for conditioned medium [62]. We subsequently showed that exosomes constitute about 10% of the conditioned medium in terms of protein amount [LAI RC, LIM SK, UNPUBLISHED DATA]. Therefore, the therapeutic activity in the hESC-MSC conditioned medium could be attributed primarily to the exosome [62]. The secretion of cardioprotective exosomes was not unique to hESC-MSCs and was also found to be produced under nonhypoxic culture conditions by MSCs derived from aborted fetal tissues [63]. Therefore, these observations suggest that the secretion of protective exosomes is a characteristic of MSCs and may be a reflection of the stromal support role of MSCs in maintaining a microenvironmental niche for other cells such as hematopoietic stem cells. The secretion of exosomes may also be a dominant function of MSCs. We recently observed that when GFP-labeled exosome-associated protein CD81 is expressed in hESC-MSCs (FIGURE 2A), they exhibit a punctate cytosolic distribution and these labeled proteins were secreted (FIGURE 2C). CD81 is a classical tetraspanin membrane protein usually found localized to the plasma membrane (as typified by their distribution in HEK 293 cells) (FIGURE 2B). The cellular distribution of the labeled CD81 in hESC-MSCs and its cellular secretion suggest that MSCs are prolific producers of exosomes, and that exosome, whose main function is to mediate intercellular communication (as discussed later), is also MSCs' vehicle of choice for intercellular communication.

What are exosomes?

Exosomes are one of several groups of secreted vesicles, which also include microvesicles, ectosomes, membrane particles, exosome-like vesicles or apoptotic bodies (reviewed in [64]). Exosomes were first found to be secreted by sheep reticulocytes approximately 50 years ago [65,66]. They have since been shown to be secreted by many cell types, including B cells [67], dendritic cells [68], mast cells [69], T cells [70], platelets [71], Schwann cells [72], tumor cells [73] and sperm [74]. They are also found in physiological fluids such as normal urine [75], plasma [76] and bronchial lavage fluid [77].

Compared with other secreted vesicles, exosomes have much better defined biophysical and biochemical properties(reviewed in [64]). They have a diameter of 40–100 nm, with a density in sucrose of 1.13–1.19 g/ml, and can be sedimented at 100,000 g. Their membranes are enriched in cholesterol, sphingomyelin and ceramide, and are known to contain lipid rafts. The presence of exposed phosphatidylserine was reported to be present on the membrane of some exosomes [78,79] and absent from others [80,81]. Exosomes contain both proteins and RNAs. Most exosomes have an evolutionarily conserved set of proteins, including tetraspanins (CD81, CD63 and CD9), Alix and Tsg101, but they also have unique tissue/cell type-specific proteins that reflect their cellular source. Mathivanan and Simpson have set up ExoCarta, a freely accessible web-based compendium of proteins and RNAs found in exosomes [82,201].

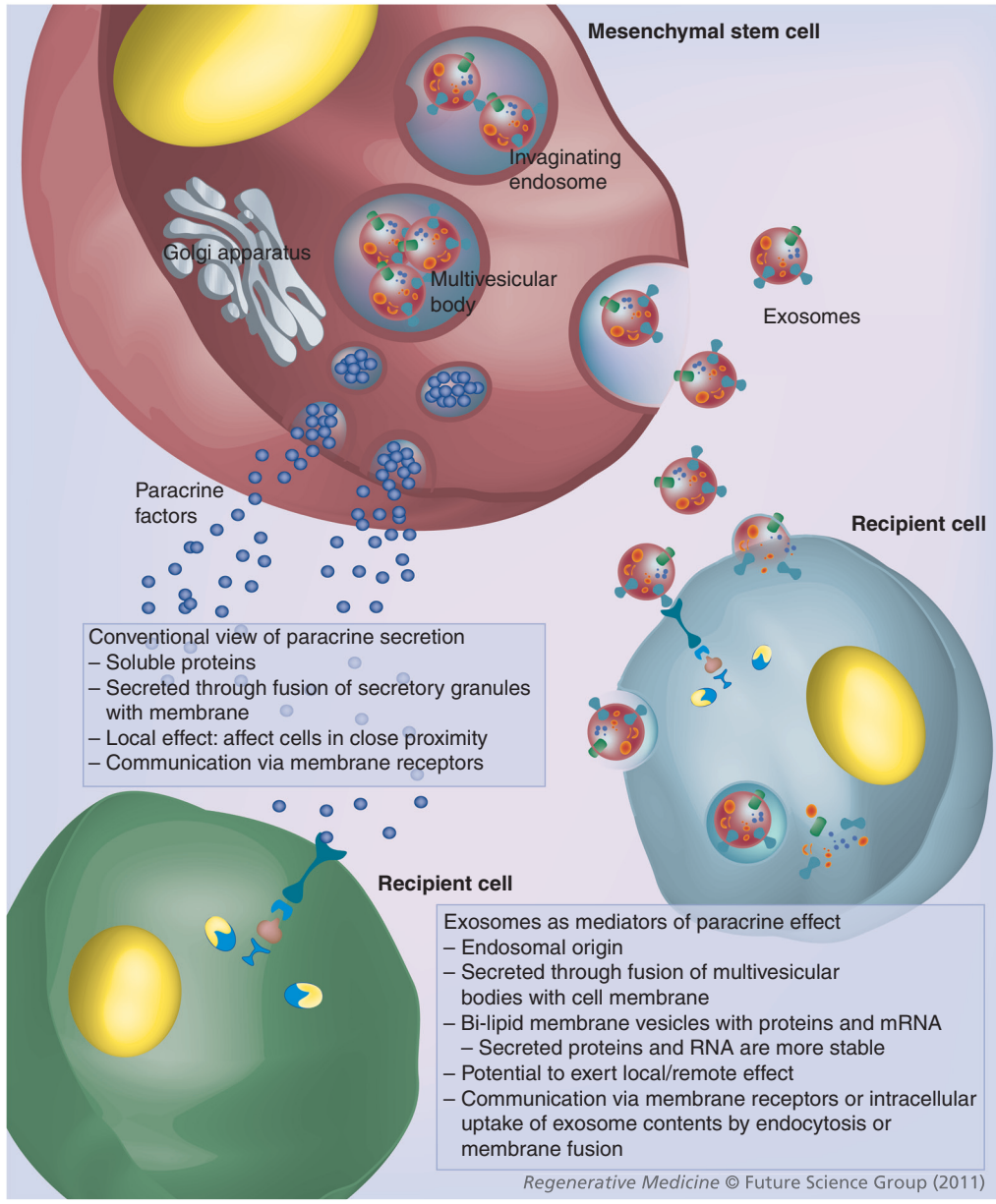

Figure 1. Paracrine effects of mesenchymal stem cells.

The functions of exosomes are not known, but they are believed to be important for intercellular communications. Exosomes were first documented in 1996 to mediate immune communication when it was observed that, when secreted by antigen-presenting cells (APCs), they bear functional peptide–MHC complexes [67]. This also provides the implication that exosomes could be used therapeutically. The therapeutic potential of exosomes was subsequently illustrated by the use of exosomes secreted by tumor peptide-pulsed dendritic cells to suppress tumor growth [68]. Ironically, exosomes are also implicated in tumorigenesis, with the observation that microvesicles mediate intercellular transfer of the oncogenic receptor EGFRvIII [83]. Exosomes have also been reported to have the potential to protect against tissue injury such as MI/R injury [62] or acute tubular injury [84].

In recent years, exosomes have also been implicated in neuronal communication or pathogenesis. For example, exosomes have been found to be released by neurons [85], astrocytes [86] and glial cells [87] to facilitate diverse functions that include removal of unwanted stress proteins [88] and amyloid fibril formation [89,90]. Exosomes containing α-synuclein have been demonstrated to cause cell death in neuronal cells, suggesting that exosomes may amplify and propagate Parkinson's disease-related pathology [91,92]. It was also reported that, in Alzheimer's disease,

β-amyloid is released in association with exosome [93]. More recently, oligodendrocytes were demonstrated to secrete exosomes to coordinate myelin membrane biogenesis [94]. Besides neuronal communication, exosomes secreted by cardiomyocyte progenitor cells were reported

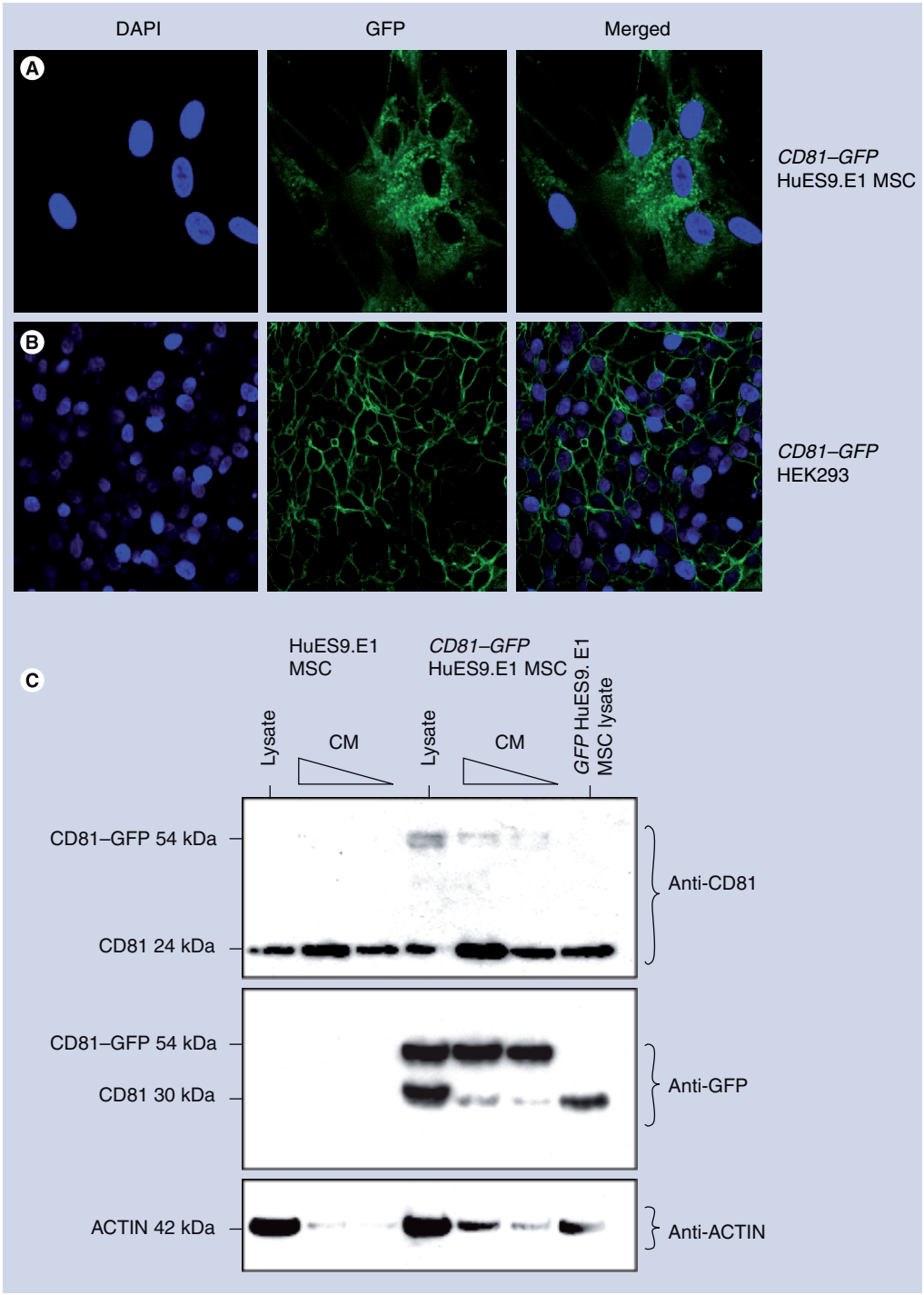

Figure 2. Expression and detection of CD81–green fluorescent fusion protein in cell lines.
(A & B) Expression of *CD81–GFP* in HuES9.E1 MSC and HEK293 cells. A *CD81–GFP* fusion gene was cloned into a pLVX-puro lentiviral expression vector to generate a CD81–GFP lentivirus. After infecting HuES9.E1 MSCs and HEK293 cells with the virus followed by drug selection, the cells were seeded onto a glass chamber slide and stained for DAPI. **(C)** Secretion of CD81–GFP fusion protein. Cell lysate and CM were prepared from HuES9.E1 MSCs and *CD81–GFP*-transfected HuES9.E1 MSCs. The cell lysate and CM were analyzed by western blot hybridization using antibodies against CD81 (top panel), GFP (middle panel) and ACTIN (bottom panel).
CM: Conditioned media; GFP: Green fluorescent protein; MSC: Mesenchymal stem cell.

to stimulate the migration of the endothelial cells [95], while those secreted by the egg facilitate the fusion of the sperm and egg [96].

Exosomes have also been implicated as a vehicle for viral and bacterial infection (reviewed in [97]), including the assembly and release of HIV [98–100] and intercellular spreading of infectious prions in transmissible spongiform encephalopathies. The association of exosomes with disease or pathological conditions makes exosomes good sentinels for diseases. It was reported that the miRNA profile of circulating exosomes could be indicative or diagnostic of ovarian cancer [101]. Similarly, the proteins in the urinary exosome have been demonstrated to reflect acute kidney injury and are candidate diagnostic markers [102]. More recently, the function of exosomes as vehicles for intercellular communication has been exploited for the delivery of therapeutic siRNAs to the brain and to provide for alternative drug delivery systems [103].

Exosome as an alternative therapeutic of MSCs?

The paracrine hypothesis introduces a radically different dimension to the therapeutic applications of MSCs in regenerative medicine. By replacing transplantation of MSCs with administration of their secreted exosomes, many of the safety concerns and limitations associated with the transplantation of viable replicating cells could be mitigated. For example, the use of viable replicating cells as therapeutic agents carries the risk that the biological potency of the agent may persist or be amplified over time when the need has been resolved, and cannot be attenuated after treatment is terminated. This could lead to dire consequences, especially if treatment was terminated as a recult of adverse outcomes. Although repeated direct endomyocardial transplantation of MSCs has been demonstrated to be relatively safe [104], intravascular administration could lead to occlusion in the distal microvasculature as a consequence of the relatively large cell size [105]. Transplantation of MSCs has been reported to induce proarrhythmic effects [106–108]. Their potential to differentiate into osteocytes and chondrocytes has also raised long-term safety concerns regarding ossification and/or calcification in tissues as reported in some animal studies [109].

Besides mitigating the risks associated with cell transplantation, exosomes can also circumvent some of the challenges associated with the use of small soluble biological factors such as growth factors, chemokines, cytokines, transcription factors, genes and RNAs [110]. The delivery of these factors to the right cell type and, in the case of those factors that work intracellularly, the delivery into the right cellular compartments, while maintaining the stability, integrity and biological potency of these factors during manufacture, storage and subsequent administration remains a costly challenge. As a bi-lipid membrane vesicle, exosomes not only have the capacity to carry a large cargo load, but also protect the contents from degradative enzymes or chemicals. For example, protein and RNA in MSC exosomes were protected from degradation by trypsin and RNase as long as the lipid membrane was not compromised [62,111]. We also found that storage without potentially toxic cryopreservatives at -20°C for 6 months did not compromise the cardioprotective effects of MSC exosomes or their biochemical activities [Lai RC, Lim SK, Unpublished Data].

Exosomes are known to bear numerous membrane proteins that have binding affinity to other ligands on cell membranes or the extracellular matrix, such as transferrin receptor, tumor necrosis factor receptors, lactadherin, integrins and tetraspanin proteins (e.g., CD9, CD63 and CD81) [82]. These membrane bound molecules provide a potential mechanism for the homing of exosomes to a specific tissue or microenvironment. For example, integrins on exosomes could home exosomes to cardiomyocytes that express ICAM1, a ligand of integrins after MI/R injury [112], or to VCAM-1 on endothelial cells [113]. Tetraspanin proteins, which function primarily to mediate cellular penetration, invasion and fusion events [114], could facilitate cellular uptake of exosomes by specific cell types.

Exosomes may also facilitate the uptake of therapeutic proteins or RNAs into injured cells. Although cellular uptake of exosomes has been demonstrated to occur through endocytosis, phagocytosis and membrane fusion [115–117], the mechanism by which these processes are regulated remains to be determined. It was observed that the efficiency of exosome uptake correlated directly with intracellular and microenvironmental acidity [117]. This may be a mechanism by which MSC exosomes exert their cardioprotective effects on ischemic cardiomyoctyes that have a low intracellular pH [118].

Despite being smaller than a cell, exosomes are relatively complex biological entities that contain a range of biological molecules, including proteins and RNA, making them an ideal

therapeutic candidate to treat complex injuries such as MI/R injury. It is well established that MI/R injury occurs paradoxically in response to a therapy that is highly effective in resolving the disease precipitating problem of no flow and ischemia. During MI/R injury, the restoration of blood and oxygen to ischemic myocardium paradoxically exacerbates the ischemia-induced cellular insults. This is because the biochemical cascades required for cell survival that are initiated by cells during no flow and ischemia [119] are not compatible with the rapid restoration of flow and oxygen supply, and at the same time, cells cannot alter their biochemical activities expeditiously enough to adapt to this restoration. This latter phenomenon was best evidenced by the reduction of MI/R through postreperfusion conditioning or postconditioning where cells were exposed to repeated short nonlethal cycles of reperfusion/ischemia to facilitate biochemical adaptation to reperfusion [120–131].

We postulate that with their complex cargo, exosomes would have adequate potential to participate in a wide spectrum of biochemical and cellular activities, and simultaneously target and correct the various ischemia-induced cascades, and prevent occurrence of the paradoxical reactions induced by reperfusion. In addition, many of the proteins in the exosomes are enzymes. Since enzyme activities are catalytic rather than stoichiometric, and are dictated by their microenvironment (e.g., substrate concentration or pH), the enzyme-based therapeutic activities of exosomes could be activated or attenuated according to the release of injury-associated substrates, which in turn, is proportional to the severity of disease-precipitating microenvironment. Resolution of the disease-precipitating microenvironment would reduce the release of injury-associated substrates and also the activity of exosome enzymes. Consequently, the efficacy of exosome-based therapeutics could be highly responsive to, but also limited by, the disease-precipitating microenvironment.

Together, the features discussed here render exosomes a highly efficacious therapeutic in neutralizing the complexity of MI/R and an effective adjuvant to complement current reperfusion therapy.

Translating hESC-MSC exosomes into therapeutics

The translation of cardioprotective MSC exosomes into a therapeutic agent presents several unique challenges. The first major challenge would be to manufacture Good Manufacturing Practices (GMP) grade exosomes from nonautologous cell sources. Although exosomes as therapeutics have already been tested as a form of cancer vaccine in the clinic [132–134], these tests were limited to exosomes produced during short-term *ex vivo* culture of autologous dendritic cells. These exosomes, also known as dexosomes, were found to be safe in the small clinical trials [132]. Unfortunately, the manufacture of these exosomes cannot provide guidance for the large-scale GMP production of exosomes from nonautologous cell sources such as exosomes from hESC-MSCs. This manufacturing process faces many unique challenges, including ethical, legal, technical and regulatory/safety issues.

The use of hESCs for the derivation of MSCs presents both ethical and legal challenges. While ethical objections to the derivation and use of hESCs have initially hindered hESC research, they have abated. Instead, the use and applications of hESCs is now being hindered by complex and widespread patenting in some countries [135] and the ban on stem cell-related patents in other countries [136]. To encourage the development of hESC-based therapeutic applications, the need for freedom to use and share hESC resources and knowledge must be balanced with a need to incentivize commercial development of stem cells by protecting the intellectual property generated from research and development efforts. Unfortunately, this balance has not yet been reached.

One of the major technical hurdles to the use of hESC-MSCs is their large but finite expansion capacity, resulting in the need for constant costly re-derivation from hESC and re-validation of each of the derived cell batch. Therefore, a robust scalable and highly renewable cell source will be central to the development of a commercially viable manufacturing process for the production of MSC exosomes in sufficient quantity and quality to support clinical testing or applications. To address this issue, we demonstrated that immortalization of the ESC-MSC by Myc did not compromise the quality or yield of exosomes [137]. Therefore, this provides a potentially inexhaustible cell source for MSC exosome production. The translation of MSC exosomes into clinical applications is also complicated by the relative novelty of exosomes with few precedents in the regulatory and safety space of biopharmaceuticals. This will require the formulation of new standards for manufacture, safety and quality control.

Future perspective

The discovery of stem cells and their potential in regenerative medicine has evoked much excitement and hope in treating some of today's most intractable diseases, including cardiac disease. However, much of the euphoria has dissipated as animal experimentation revealed and identified potential problems in translating the use of stem cells to treat cardiac disease. Although the reproducible large-scale preparation of homogenous clinically compliant 'normal' healthy cells has been a major preoccupation in the development of stem cell-based therapies in general, this has proven not to be an impediment in the development of such therapies for cardiac disease, as evidenced by the large number of stem cell-based clinical trials that are already being conducted. Instead, the problems facing stem cell-based therapies for cardiac disease are potentially more insidious. At present, most of the stem cells used in clinical trials are MSCs and bone marrow mononuclear cells that are generally considered to be safe. However, despite eliciting a sometimes positive therapeutic response, these cells often do not integrate or persist in the heart tissues. By contrast, the use of myogenic cells, such as

Executive summary

Stem cells in the treatment of acute myocardial infarction

- Advances in reperfusion therapy have increased survival of patients with severe acute myocardial infarction and contributed to a growing epidemic of heart failure.
- As reperfusion therapy itself causes lethal injury and has been demonstrated to be intractable to pharmaceutical intervention, stem cells are being scrutinized as alternative therapeutic agents.
- Attempts using stem cells to treat heart disease have generated mixed outcomes.
- Transplantation of bone marrow stem cells generally improved cardiac functions with little evidence of engraftment and differentiation of the transplanted stem cells.
- Effects of stem cell transplantation have been attributed to secretion of paracrine factors by the transplanted stem cells.

Mesenchymal stem cells & the treatment of cardiovascular disease

- Animal studies and early clinical trials demonstrated that mesenchymal stem cell (MSC) transplantation improved cardiac function after myocardial infarction.
- Inefficient MSC engraftment and differentiation, and their rapid cardioprotective effects suggested that MSCs act via a secretion-based paracrine effect rather than a cell replacement effect.

Paracrine secretion of MSCs

- MSCs synthesize a broad spectrum of growth factors and cytokines that exert paracrine effects.
- Gnecchi *et al*. produced the first evidence that cellular secretion alone improved cardiac function in an animal model of acute myocardial infarction.
- Culture medium conditioned under nonhypoxic conditions by untransformed MSCs derived from human embryonic stem cells or aborted fetal tissues reduce infarct size in animal models of myocardial ischemia/reperfusion.
- Exosome is the primary mediator of MSCs' paracrine effect.

What are exosomes?

- Exosomes are bi-lipid membrane vesicles secreted by many cell types into culture medium and other bodily fluids such as blood and urine.
- They function as mediators of intercellular communication.

Exosome as an alternative therapeutic for MSC?

- Exosome-based therapy circumvents some of the concerns and limitations in using viable replicating cells and does not compromise some of the advantages associated with using complex therapeutic agents such as cells.
- Exosomes are ideal therapeutic agents because their complex cargo of proteins and genetic materials has the diversity and biochemical potential to participate in multiple biochemical and cellular processes, an important attribute in the treatment of complex disease.
- Exosomes home to specific tissue or microenvironment.
- Their bi-lipid membranes can protect their biologically active cargo allowing for easier storage of exosomes, which allows a longer shelf-life and half-life in patients.
- Their biological activities are mainly enzyme-driven and, therefore, their effects are catalytic and not stoichiometric.
- Having enzyme-driven biological activities, they are dependent on the microenvironment (e.g., substrate concentration or pH) and could be activated or attenuated in proportion to the severity of disease-precipitating microenvironment.
- Exosome-based therapy cannot replace lost myocardium but can prevent or delay loss of myocardium.

Challenges for translating embryonic stem cell-MSC exosomes into therapeutics

- Ethical issues exist, especially with the derivation and use of human embryonic stem cells for generating MSCs.
- Legal issues include excessive intellectual property protection in some countries, which hinder research and development. A ban on embryonic stem cell-related intellectual property in other countries de-incentivize research and development.
- Technical limitations include the need for a robust scalable and highly renewable cell source embryonic stem cell-MSCs to support large scale, commercially viable manufacturing process.
- Exosomes are relatively novel biological entities with few precedents to establish safety and manufacturing guidance.

skeletal myoblasts, cardiac progenitors or stem cell-derived cardiomyocytes, to replace lost myocardium has been demonstrated to increase the risk of arrhythmias when the donor cells failed to couple with the host tissues, in early clinical trials and animal studies. Resolution of these problems would require the development of cell delivery or cell engraftment techniques that can facilitate proper mechanistic integration of the donor cells into the recipient tissues to enable coordinated heart functions. Other potential problems include problems that are generally universal in cell-based therapy, such as host rejection and risk of tumor formation. We anticipate that aside from the issue of proper integration of donor cells into the recipient heart, many of these problems will be resolved or partially resolved in the next 5–10 years. However, without the resolution of the poor coupling between donor and recipient cells, it is unlikely that cell-based therapy using stem cells to replace lost myocardium will evolve into a standard therapy for the treatment of cardiac disease. Although paracrine secretion of stem cells provides an alternative approach for the development of stem cell-based therapies, it does not replace the need for cell-based therapy to replace lost myocardium. However, it may reduce cardiac injury and delay the loss of myocardium to the extent that replacement of lost myocardium does not become critical. The identification of exosomes as the cardioprotective factor in MSC secretion reduces the paracrine secretion to a single biological entity that is more amenable to the stringent criteria for clinically compliant preparation and use. As a bi-lipid membrane vesicle with many membrane-bound proteins and a diverse cargo, exosome represents an ideal therapeutic agent that has the potential to home to target tissues and treat complicated diseases such as MI/R injury. With the advance of new bioengineering and cellular modification techniques, engineering or modification of the exosome surface antigen and internal content will enable it to target other more complex diseases with even more specificity.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

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