

Review Article

MSC exosome works through a protein-based mechanism of action

Wei Seong Toh^{1,2,*}, Ruenn Chai Lai^{3,*}, Bin Zhang³ and Sai Kiang Lim^{3,4}

¹Faculty of Dentistry, National University of Singapore, Singapore; ²Tissue Engineering Program, Life Sciences Institute, National University of Singapore, Singapore; ³Institute of Medical Biology, Agency for Science, Technology and Research, Singapore; ⁴Department of Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

Correspondence: Sai Kiang Lim (saikiang.lim@imb.a-star.edu.sg)



Mesenchymal stem cell (MSC) exosome specifically defines the 50–200 nm vesicles that are secreted into the extracellular space when multivesicular bodies in the MSC fuse with the plasma membrane. However, the exosome is just one of several 50–200 nm extracellular vesicles (EVs) known to be secreted by cells. Nevertheless, the term ‘MSC exosome’ is often used to describe populations of 50–200 nm EVs that are prepared from culture medium conditioned by MSCs on the basis that these populations collectively exhibited typical exosome-associated proteins such as endosomal proteins, TSG101 and Alix, and tetraspanin proteins, CD9, CD63 and CD81. They also carry a rich diverse RNA cargo. MSC exosomes are increasingly implicated as the mediator of many of the MSC-associated therapeutic potencies. They elicit therapeutic activity by delivering their cargo of potentially therapeutic proteins and RNAs to the recipient cells. The therapeutic potency of MSC exosomes is usually rationalized on the presence of a biologically relevant protein or RNA in the MSC exosome. In the present paper, we expanded this rationale beyond a physical presence to include biologically relevant concentration, biochemical functionality and the potential to elicit an appropriate timely biochemical response. Based on these, we propose that MSC exosomes most probably work through the protein rather than the RNA.

Mesenchymal stem cell

Mesenchymal stem cells (MSCs) are the most extensively used stem cells in the field of regenerative medicine. From 2006 to 2012, the MSC-based product FDA IND submissions increased by three-fold [1]. A simple search using keywords ‘mesenchymal stem cell’ or ‘mesenchymal stromal cell’ in clinicaltrials.gov database uncovers more than 800 clinical trials (<http://www.clinicaltrials.gov/>, accessed on January 2018). The major reasons for their popularity are the widely reported therapeutic efficacy against many diseases in animal models, easy accessibility from many adult tissues such as bone marrow aspirate and medical waste tissues such as umbilical cord, large *ex vivo* expansion capacity and an immune-privileged status that renders MSCs amenable to allogeneic transplantation.

The use of MSCs as therapeutics was initially rationalized on their large *in vitro* differentiation potential to generate many different cell types that could replace lost cells in injured or dead tissues [2]. However, several critical observations in the field have challenged this rationale. It was frequently observed that functional improvement after MSC transplantation does not correlate with engraftment or differentiation of MSCs [3–5]. For example, in a porcine myocardial infarction study where it was observed that MSC engraftment in the infarct zone was maximal with intracoronary delivery and minimal with intravenous delivery, the relative infarct sizes in the animals were similar and independent of the delivery mode [6]. In addition, functional recovery also appeared to precede differentiation of MSCs. Toma et al. [7] have reported transplanted MSCs differentiated into cardiomyocytes only

*Co-first authors.

Received: 4 April 2018

Revised: 24 May 2018

Accepted: 29 May 2018

Version of Record published:

9 July 2018

after 4 days, and Dai et al. [8] observed the transient left ventricular function improvement in animals after MSC transplantation disappeared as the transplanted cells start to acquire cardiac markers. Still others observed cardiac improvements in the animals even when they could not detect the presence of transplanted MSCs in the heart [9]. Gneocchi et al. [10] also reported improved ventricular function within 72 h after transplantation long before transplanted MSCs are known to differentiate into cardiomyocytes. Also, transplanted MSCs could improve cardiac function even if most of the transplanted cells engraft in the lungs [8,9,11–13]. Together, all these observations and studies contradict the ‘engraft-and-differentiate’ hypothesis for the efficacy of transplanted MSCs. These divergent observations led increasingly to the proposal that MSCs reduce injury and support tissue repair through their secretion [14]. Indeed, as early as 2004, Kinnaird et al. [15] reported that culture medium conditioned by MSCs improved collateral flow recovery and remodeling, and improved limb function in a mouse model of hindlimb ischemia.

In 2006 [10] and 2008 [16], Gneocchi et al. and our group independently showed that MSC secretion reduced infarct size in a mouse model of acute myocardial infarction in the absence of the MSC itself. These studies confirm the hypothesis that MSC secretion plays an important role in MSC therapeutic effect and further testing of this hypothesis led to the discovery of extracellular vesicles (EVs) as the mediating factor in MSC secretion [17]. In 2009, Camussi’s group first showed that MSC exerts its therapeutic efficacy through EVs. Specifically, they showed that 80–1000 nm EVs, namely microvesicles, protect against acute tubular injury [18]. In 2010, our group further showed through size fractionation studies that the 50–200 nm EVs known as exosomes were efficacious against myocardial reperfusion injury [19].

Exosomes

Exosome is the best defined secreted vesicle among the different EV types reported to date, namely microvesicles, microparticles, ectosomes, shedding particles or apoptotic bodies [20]. Physically, they are 40–150 nm in diameter with a bi-lipid membrane that has the same orientation as the plasma membrane. The size of exosomes varies according to the methodology used. In general, sizes estimated by electron microscopy are usually smaller due to dehydration during sample preparation. With methodologies such as dynamic light scattering or nanoparticle tracking analysis where the samples were not dehydrated, the sizes are larger and in the range of 100–200 nm. Like a cell, the exosome membrane is enriched in signaling molecules and surface antigens, and it reportedly contains both proteins and genetic materials but does not contain any organelle such as nucleus or mitochondrion. The defining property that distinguishes exosomes from the other EV types is its endosomal origin.

Exosomes are formed when the membrane of endosome invaginates to produce the multivesicular body (MVB). They are released from the cells when the MVB fuses with the cell membrane [21]. Exosomes have a flotation density of 1.1–1.18 g/ml. Consistent with its endosomal origin, the exosome membrane is enriched in cholesterol, sphingomyelin and ceramide that are typical of lipid rafts [22,23]. Generally, all exosomes share a common set of proteins, namely tetraspanins (CD81, CD63 and CD9), Alix, TSG101, but they also contain specific proteins that reflect the origin and the pathophysiological states of the cell source [24]. According to Exocarta, the 25 proteins most commonly found in exosomes are HSPA8, CD9, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ACTB, CD63, CD81, ANXA2, enolase 1 (ENO1), HSP90AA1, EEF1A1, PKM2, YWHAE, SDCBP, PDCD6IP, ALB, YWHAZ, EEF2, ACTG1, LDHA, HSP90AB1, ALDOA, MSN, ANXA5, phosphoglycerate kinase 1 (PGK1) and CFL1 (http://exocarta.org/exosome_markers).

From its early days as ‘garbage bags’ to dispose of unwanted materials like transferrin receptors during the maturation of reticulocytes to red blood cells [25], the exosome has come a long way and is now highly appreciated as an efficacious vehicle for intercellular communication to modulate recipient cell biology. As nearly all cell types are reported to secrete and take up exosomes, secretion and uptake of exosomes are likely to be conserved cellular functions and thus have universally important functions. Consistent with its role as an intercellular communication vehicle, the exosome reportedly participates in many different biological processes such as adipose tissue regeneration [26], T-cell stimulation [27], myelin membrane biogenesis [28], innate immunity modulation [29], endothelial cell migration [30], neurotransmission [31], tumor growth suppression [32], glioma growth inhibition [33], and egg and sperm fusion [34]. However, the efficacy of exosomes as vehicles for intercellular communication also has the undesirable consequence of being efficacious in disseminating pathological materials such as the propagation of prion diseases [35], HIV [36], hepatitis C virus [37] and cancer [38,39].

Discovery of therapeutic MSC exosome

Therapeutic MSC exosome was first described in 2010 [19]. When a culture medium conditioned by MSCs was fractionated by size exclusion using high-performance liquid chromatography, the conditioned medium was found to contain a fraction of homogeneously sized particles with a hydrodynamic radius of 55–65 nm. Under the electron microscope, these particles have a smaller diameter of 40–100 nm, due possibly to dehydration during sample preparation. These particles collectively exhibited distinct exosome features, namely endosomal markers ALIX and TSG101, tetraspanin proteins, CD9 and CD81, RNA, flotation density of 1.1–1.18 g/ml and cholesterol-rich lipid membrane [19,40]. They were efficacious in reducing infarct size in a rodent model of myocardial ischemia/reperfusion (I/R) injury.

MSC exosome as the paracrine mediator of MSC therapeutic potency

Since the first report, MSC exosome has been reported to be therapeutically efficacious against liver fibrosis [41], retinal laser injury [42], limb ischemic injury [43], diabetes-induced cognitive impairment [44] and inflammation-induced preterm brain injury [45]. MSC exosomes were also reported to be efficacious in enhancing neurovascular plasticity after stroke [46], dormancy in metastatic breast cancer cells [47], hepatic regeneration in drug-induced liver injury [48], skeletal muscle regeneration [49], cutaneous wound healing [50], fracture healing [51] and cartilage regeneration [52–54]. This range of exosome activities mirrors the reported wide-ranging therapeutic efficacy of MSCs, suggesting that the therapeutic efficacy of the MSC is largely underpinned by its exosomes.

MSC exosome as a ‘cell-free’ therapeutic

The discovery that exosomes mediate the wide-ranging therapeutic efficacy of MSCs eliminates many challenges associated with the use of living replicative cells as it radically transforms a living cell-based MSC therapy to a non-living MSC therapeutic. While MSC therapies are generally considered safe as documented in several studies [55–57], the use of relatively large living MSCs carries inherent risks such as occlusion in microvasculature [58], transformation of transplanted cells into inappropriate cell types or cancer [59] and pro-arrhythmic side effects [60–62]. The capacity of MSCs to differentiate into chondrocytes and osteocytes also raised long-term safety concerns regarding ectopic ossification and/or calcification in tissues as reported in some animal studies [63]. Transplantation of living replicative cells is also risky as the grafted cells cannot be removed in events of adverse response or upon disease resolution. Operationally, the manufacture of cell-based MSC therapeutics also poses significant challenges as cell viability, potency and transformation are difficult to monitor and maintain in the manufacturing process, storage and delivery to the patient.

In contrast with large living cells, nanosized, non-living exosomes would not occlude microvasculature, transform into inappropriate cell types or persist as permanent grafts upon cessation of therapy. Exosome manufacture is also scalable and more amenable to process optimization as the producer cell source could be clonally selected, derived and immortalized to ensure standardized and reproducible production of exosomes [64]. All in all, MSC exosome-based therapeutic could potentially be a safer, cheaper and more effective treatment modality than cell-based MSC therapeutics.

Definition of MSC exosome

MSC exosomes are currently prepared according to size or density. Such methods cannot differentiate exosomes from other EVs or biological macromolecules such as lipoprotein complexes, protein aggregates and apoptotic bodies. MSC exosome preparation is invariably described as a preparation of 100–200 nm particles with a density of 1.10–1.18 g/ml and collectively carries exosome-associated markers such as the tetraspanin proteins and RNAs. The endosomal biogenesis of MSC exosomes is implied by the presence of endosomal markers, ALIX and TSG101. However, such parameters are descriptive and are not amenable as references for batch comparison. In addition, it has been recently shown that such preparations could be highly heterogeneous and could contain three or more distinct EV populations [65].

Each of these three EV populations can be differentiated by their affinity for different membrane lipid-binding ligands, namely cholera toxin B chain (CTB), annexin V (AV) and Shiga toxin B chain (ST), which binds GM1 ganglioside, phosphatidylserine and globotriaosylceramide (or Gb3). As each of these ligands are multivalent, i.e. binding multiple lipid molecules, this ligand–lipid binding is likely to occur only at sites of

high local lipid concentration where the lipids are in sufficiently close proximity to enable multivalent binding. These sites would probably represent the sites of biogenesis of the respective EVs. Binding sites for CTB, AV and ST were observed to localize to the plasma membrane and cytoplasm, cytoplasm and nucleus, respectively.

The localization of CTB to the plasma membrane and cytoplasm is consistent with the enrichment of CTB-binding GM1 gangliosides in lipid rafts which are the major site for receptor-mediated endocytosis and biogenesis of endosomes, and consequently, also exosomes. Based on several lines of evidence including pulse-chase studies, we demonstrated that CTB-binding EVs are exosomes, i.e. EVs with an endosomal biogenesis [66]. The AV-binding sites in the cytoplasm have not been identified yet. Because of the multivalency of AV binding, the AV-binding sites are likely to represent sites of high local phosphatidylserine concentration. Unlike the co-localization of CTB-binding sites with CD81, there is little co-localization of AV-binding sites with CD81 or the plasma membrane, suggesting that in healthy cells, the AV-binding EVs are not exosomes or apoptotic bodies. ST-binding sites are localized primarily in the nucleus, suggesting that the ST-binding EVs originate from the nucleus. The cargo of each of the three EV types is consistent with the subcellular ligand-binding sites. The CTB-binding EVs carry most of the exosome-associated proteins of the MSC exosome preparation. Other than actin which is present in all three EV types, we have not yet identified any proteins that are predominantly associated with AV-binding EVs. Among the three EV types, only the ST-binding EVs carry RNA and fibronectin.

Consequently, most and probably all MSC exosome preparations reported to date are likely to be heterogeneous populations of small EVs that also included exosomes. In this manuscript, the term 'MSC exosome' shall refer to EVs of 50–200 nm that are secreted by MSCs.

RNA cargo of MSC exosome preparation

MSC exosomes like most exosomes carry an RNA cargo. Unlike several reports on RNA in exosomes, we observed that the RNA in MSC exosomes were relatively short and when visualized on agarose or polyacrylamide gels, most of the RNAs are less than 300 nucleotides (nts) [40]. Unlike other exosomes, 18S or 28S RNAs were not detectable in MSC exosomes [67,68]. RNA in the MSC exosome was susceptible to enzymatic RNase digestion only if the exosome preparation was pre-exposed to an SDS-based lysis buffer, cyclodextrin (a cholesterol chelator) or phospholipase A2. It was reported that only a fraction of the miRNAs identified in MSCs were secreted in MSC exosomes, suggesting that miRNA secretion by MSCs is a regulated process [40]. Significantly, many of the miRNAs were pri- and pre-miRNAs, but not mature miRNAs. However, miRNA independent of its state of processing is not the major RNA species in MSC exosomes.

Deep sequencing, which presently represents the state of the art for non-biased DNA and RNA analysis, revealed MSC exosomal RNA as a highly heterogeneous RNA population of ~100 nts [65]. We observed that of a total of 151.13 million reads generated by RNAs in MSC exosomes, only 1.4 million reads or 0.9% were miRNAs. Considering that miRNA constitutes ~0.01% of total cellular RNA by weight [69], the miRNA content in MSC exosome preparation was enriched 10 times. The majority of the RNAs in the exosomes mapped to ribosomal RNAs, Y RNA, snoRNA, protein-coding RNA, snRNA and processed transcripts. As the average RNA length in exosomes is ~100 nts, most of the exosome RNAs, except the miRNAs, are essentially fragments of the RNAs to which they are mapped, and are not likely to elicit any biological functions. Baglio et al. [70] also observed in their deep sequencing of MSC exosome RNA that miRNAs constituted 2–5% of the RNA and the majority of which are tRNAs. Of the miRNAs detected through RNA sequencing, both groups independently identified 111 miRNAs [65,70].

The RNA cargo of exosome has generated much research interest because it was reported that exosomes carry mRNA and microRNA that could be delivered to other cells where the RNA elicits biological responses from the recipient cells [68,71]. However, subsequent analysis revealed that RNAs in most exosomes are short and are in the range of 200–400 nts [40,72–76]. As the average human mRNA is ~2314 nts [77], RNA in exosomes are generally too short to carry protein-coding information. As such, the therapeutic effects of MSC exosomes have been increasingly attributed to the intercellular transfer of miRNA. It should be noted that MSCs are known to also transfer miRNAs to other cells via the gap junction-mediated import, which was reportedly more efficient [78].

As miRNAs are biologically functional only when associated with RNA-induced silencing complex (RISCs), and only pre-miRNA could be loaded onto RISCs, miRNA-induced biological effects of MSC exosomes would have to be mediated by either pre-miRNA or RISC-loaded mature miRNA [79]. Hence, it is imperative to specify if the exosome-associated miRNA of interest is a pri-, pre- or mature miRNA. If the miRNA of interest

is a mature miRNA, it should be determined if the miRNA was associated with an RISC. A search of the Exocarta and Vesiclepedia (accessed 17 January 2018) revealed that Dicer and Argonaute are not frequently detected in exosomes or EVs. For example, of the 41 860 protein entries in Exocarta, there were only six entries of Argonaute protein and none for Dicer protein. Similarly, of the 92 897 protein entries in Vesiclepedia, there were two for Dicer and none for Argonaute. Interestingly, none of the eight entries were in MSC exosomes or EVs. This was consistent with an earlier report that Dicer and Ago2 were not detectable in MSC exosomes [40]. It has also been reported that the majority of circulation miRNAs are non-vesicle-associated ribonucleo-protein complexes [80], raising a possibility that some of the EV-associated miRNAs may be non-vesicle-associated ribonucleoprotein complexes that are co-purifying with exosomes and small EVs. Together, these observations demonstrate that exosomes or EVs do not carry intact RISCs.

miRNA as mediators of MSC exosome therapeutic efficacy

The wide repertoire of miRNAs in MSC exosomes could conceivably provide an miRNA-based mechanism for the wide-ranging therapeutic effects of MSC secretion. The intercellular transfer of miRNA via exosomes resulting in functional activity in recipient cells was first observed in Epstein–Barr virus-infected cells where viral miRNAs were transferred to uninfected recipient cells [32] and also between COS-7 cells and metastatic prostate cancer cells [73]. MSC exosome miRNAs have been implicated in many MSC exosome-mediated cellular activities such as angiogenesis and anti-angiogenesis, immunomodulation, anti-apoptosis and anti-fibrosis as reviewed by Katsuda and Ochiya [81]. For instance, MSC exosomes reportedly transfer miRNAs such as miR-125a and miR-30b to promote tube formation of human umbilical vein endothelial cells through suppression of the angiogenic inhibitor delta-like 4 (DLL4) [82,83]. Interestingly, MSC exosomes also contain anti-angiogenic miRNAs such as miR-16 [84] and miR-100 [85] that suppress angiogenesis by targeting vascular endothelial growth factor (VEGF) in breast cancer cells within a tumor microenvironment. Exosomal miR-181c in human umbilical cord MSC exosomes was found to attenuate burn-induced inflammation by suppressing the Toll-like receptor-4 signaling pathway [86], and in MSCs pretreated with interleukin-1 β , exosomal miR-146a was found up-regulated and enhanced macrophage polarization to M2 macrophages that in turn attenuated inflammation and improved survival in septic mice [87]. In other studies, exosomal miRNAs including miR-19a [87] and miR-233 [88] were identified to contribute to anti-apoptotic effects of MSC exosomes in cardioprotection against hypoxia-induced myocardial I/R injury and surgically induced sepsis, respectively. Exosomal miRNAs also contribute to anti-fibrotic effects of MSC exosomes, and overexpression of miRNA-181-5p [89] and miR-122 [90] was recently found to enhance the therapeutic efficacy of MSC exosomes in CCl₄-induced liver fibrosis. Similarly, umbilical cord MSC exosomes were found to reduce scar formation during wound healing of skin defects in mouse, and this was attributed to roles of exosomal miRNAs (miR-21, miR-23a, miR-125b and miR-145) in suppressing myofibroblast formation by inhibiting TGF- β 2/Smad2 signaling [91].

Assessing miRNA-mediated mechanism of action

To determine if exosome miRNA plays a role in mediating the therapeutic efficacy of MSC exosomes, a necessary prerequisite is the presence of a biologically relevant concentration of the miRNA. To date, we have not found a report on RNA yield of MSC exosomes. In our hands, 100 μ g of MSC exosome protein or $1\text{--}3 \times 10^{10}$ particles yield $\sim 7 \mu$ g of RNA (unpublished data). This is higher than the reported yield of 2–10 ng of RNA from exosomes derived from 4 ml of serum where 1 ml of serum was determined to have $1\text{--}3 \times 10^{12}$ exosomes per ml [92]. The authors also estimated that theoretically, each exosome of 30–150 nm can accommodate $\sim 70\text{--}25\,000$, 100 nt RNA or 50 kDa protein molecules. However, based on their experiments, they estimated that an average serum-derived exosome has one or less RNA molecule. As such, our yield of 7 μ g of RNA per 100 μ g of MSC ($\sim 10^{10}$ particles) exosome protein is relatively high.

Based on RNA sequencing that is discussed earlier, $\sim 0.9\%$ of the total RNA in MSC exosome was miRNA [65]. Therefore, the amount of miRNA per 100 μ g of MSC exosomes would be 0.06 μ g (60 ng). As exosomes or EVs generally do not have RISC components and the miRNAs in MSC exosomes were enriched in precursor form [40], any miRNA effects would have to be exerted through pre-miRNAs. As pre-miRNAs have a median length of 83 nts [93] with an average molecular weight of 26 761, the number of miRNA molecules per 100 μ g of MSC exosomes is $0.6 \times 10^{-9} \times 6.02 \times 10^{23} \div 26\,761 = 1.3 \times 10^{10}$ or 1.3 miRNA per exosome. As more than 100 miRNAs have been detected in the MSC exosome, the probability of an exosome having a specific miRNA is 1 in 100. This distribution of miRNA in exosomes is consistent with that described by Chevillet et al. [94] who estimated that, on average, there was far less than one molecule of a given miRNA per exosome, even for

the most abundant miRNAs in exosome preparations. Thus, a cell would have to take up at least 100 exosomes for one given miRNA molecule. As the number of miRNA molecules per cell varies widely from 115 330 copies of miRNAs per cell in mouse liver to 11 587 miRNAs per cell in highly purified CD34+/CD133(-) human hematopoietic progenitor cells (HSCs) [95] and the median copy number for a specific miRNA in CD34+/CD133(-) HSCs is 178 copies per cell, the uptake of one miRNA by a cell is not likely to elicit a cellular response.

We next evaluate the viability of an miRNA-based mechanism of action (MOA) for the MSC exosome by comparing with siRNA, a similar RNA biologic. Intravenous exosome doses in mice range from 1 to 100 μg /mouse, and this would be equivalent to 0.6–60 ($7/100 \times 0.9\% \times 1$ to $7/100 \times 0.9\% \times 100$) ng/mouse. In comparison, the reported therapeutic doses for double-stranded 21 mer siRNA are much higher. For example, sub-retinal doses of VEGF siRNA to inhibit ocular vascularization in mice were 20 pmol [96] which translates to 277.2 ng per retinal. To relieve neuropathic pain, rats were dosed intrathecally at 400 $\mu\text{g}/\text{day}$ P2X₃ siRNA [97] which would be ~ 40 $\mu\text{g}/\text{day}$ for mice on a weight equivalent dose where a rat is assumed to be 10 times heavier than a mouse. Even when siRNAs were administered using delivery vehicles such as lipid nanoparticles, the doses were 2.0 or 4.0 mg kg⁻¹ siRNA [98] or 50–100 $\mu\text{g}/25$ g mouse. On average, the miRNA in one therapeutic exosome dose is at least 10³ lower than an average siRNA dose.

In summary, our analysis here demonstrates that the presence of a specific miRNA is not sufficient to implicate a mechanistic role without due consideration of its concentration, the structure of the miRNA and the availability of accessory proteins such as RISC components.

Protein cargo of MSC exosomes

To date, several groups have reported on the proteome of MSC exosomes [99–104]. More than a thousand proteins have been identified. Mapping of the proteome to biological processes revealed that MSC exosome proteomes are involved in many key biological processes that are important in cellular communication, cellular structure, inflammation, exosome biogenesis, development, tissue repair and regeneration, and metabolism [104]. Like miRNA in MSC exosomes, proteins in MSC exosomes have the potential to modulate many of the biological processes that are involved in disease pathogenesis or tissue repair and regeneration.

Assessing protein-based MOA

As with miRNA, a minimal requisite for a protein-based MOA is the presence of a biologically relevant level of the protein in a therapeutic dose of the exosome. It is critical that the protein should have sufficient biochemical potency in that therapeutic dose to elicit a relevant biochemical effect. We had previously proposed that MSC exosomes reduced infarct size in a mouse model of myocardial I/R injury [19,105] and oligomycin-treated cells through a protein-mediated mechanism [101].

During I/R injury, heart tissues underwent significant proteomic changes [106]. Specifically, the key rate-limiting enzymes in fatty acid oxidation, glycolysis and tricarboxylic acid (TCA) cycle were greatly depleted, while the pro-apoptotic proteins were substantially increased after ischemia and for up to 120 min after reperfusion. These proteomic changes are consistent with reduced ATP production and increased apoptosis in myocardial I/R injury. The reduced ATP production and increased apoptosis in myocardial I/R injury could be alleviated by MSC exosomes [107]. We had attributed the MSC-mediated alleviation of reduced ATP production to the presence in the MSC exosome of all five enzymes in the ATP-generating stage of glycolysis, namely GAPDH, PGK, phosphoglucomutase (PGM), ENO and pyruvate kinase m2 isoform (PKm2). The enzymatic activity of the two ATP-generating glycolytic enzymes, PGK and PKm2, was determined to be 3.59×10^{-3} U and 5.5×10^{-3} U/ μg of MSC exosome protein, respectively [108] where one unit (U) of enzyme activity is defined as the activity required for the production of 1 μmol of product per minute. Therefore, the two ATP-producing glycolytic enzymes, PGK and PKm2 in 1 μg of MSC exosome protein, would have a combined enzyme activity of 9.1×10^{-3} U (3.59×10^{-3} U + 5.5×10^{-3} U) and could generate 9.1×10^{-3} μmol of ATP per minute. As the concentration of ATP in mammalian tissues is estimated to be 2–6 $\mu\text{mol}/\text{g}$ tissue [109] and 1 g tissue has $\sim 10^8$ cells [110], one cell has $\sim 2\text{--}6 \times 10^{-8}$ μmol ATP. Therefore, 1 μg of exosome in 1 min can generate the same amount of ATP as in $1.5\text{--}4.6 \times 10^5$ cells. Alternatively, it has been estimated that an average human cell consumes 10^9 ATP per second [111]. As two glycolytic enzymes in 1 μg of MSC exosome could potentially generate 9.1×10^{-3} μmol of ATP per minute, 1 μg of MSC exosome would generate 0.15×10^{-3} μmol or $0.15 \times 10^{-3} \times 10^{-6}$ mol of ATP per second; 1 μg of exosome could meet the ATP consumption requirements of $(6.02 \times 10^{23} \times 0.17 \times 10^{-9} \div 10^9) = 1 \times 10^5$ cells/s.

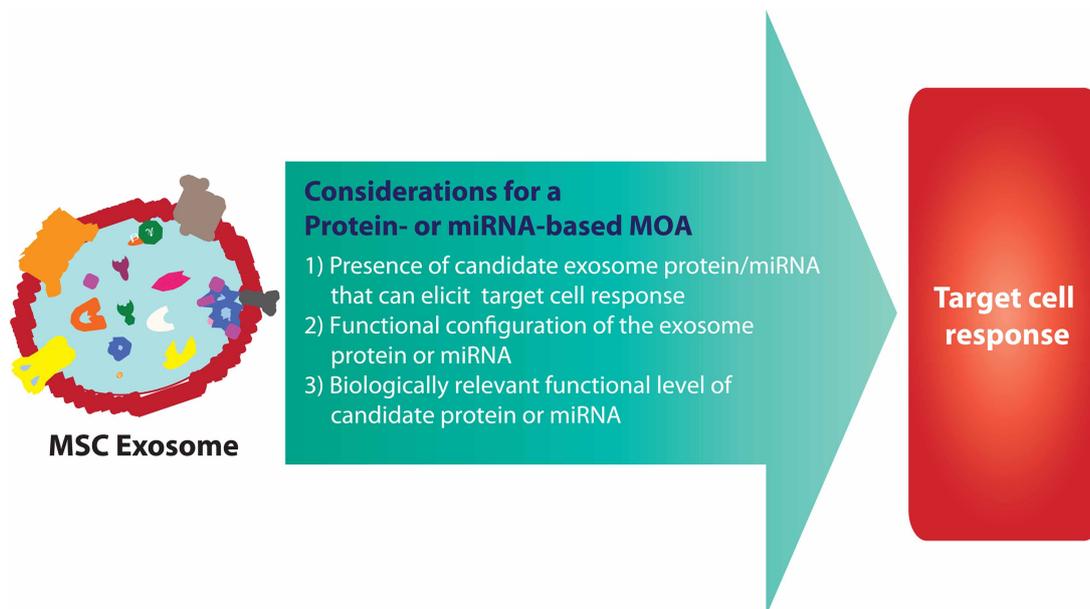


Figure 1. The MOA underpinning the therapeutic efficacy of MSC exosomes could be attributed to their cargo of protein or RNA.

As a first requisite to identifying the candidate molecule, the candidate protein or miRNA that has the potential to elicit the therapeutic response in the target cells must be present in the exosomes. Second, the candidate must be in a biologically functional configuration. In the case of protein enzyme, this property could be directly assessed by enzyme activity. For miRNA to be functional, the exosomal miRNA must either be mature miRNAs in RISCs or pre-miRNAs that could be loaded into RISCs. Third, the candidate protein or miRNA in a therapeutic dose must have sufficient functional activity to elicit the biological response of the target cell.

miRNA- versus protein-based MOA

In comparing the capacity of MSC exosome miRNAs versus proteins in eliciting biologically relevant activity, it is obvious that biologically important miRNAs are not likely to be present in either the right configuration or concentration. Specifically, mature miRNAs in MSC exosomes are not likely to be in RISCs as the RISC components are not generally present in the exosomes. On the other hand, pre-miRNAs are not present in sufficient quantities in a typical exosome dose to elicit a biologically relevant response. Unlike miRNA, proteins in a typical therapeutic MSC exosome dose have the potential to elicit a biologically relevant response as illustrated by the example of glycolytic enzymes to produce ATP. Therefore, a protein-mediated MOA represented a more likely mode of action for the exosomes. However, it should be noted that the potential to elicit a biologically relevant response is due, in large part, to the catalytic activity of enzymes, and that structural proteins may not elicit a similar biological response.

Conclusions

Although both miRNAs and proteins in MSC exosomes have the potential to biologically influence disease- or injury-associated processes, their role in mediating MSC exosome-mediated therapeutic activity has to be considered beyond a presence in the exosomes. Critical considerations as discussed here include the potential of the miRNAs or proteins in a therapeutic dose of exosome to elicit a biologically relevant activity (Figure 1). Based on these considerations, proteins could be the main driver of MSC exosome therapeutic activity.

Abbreviations

AV, annexin V; CTB, cholera toxin B chain; EBV, Epstein–Barr virus; ENO1, enolase 1; EVs, extracellular vesicles; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSCs, hematopoietic progenitor cells; I/R, ischemia/reperfusion; MOA, mechanism of action; MSC, mesenchymal stem cell; MVBs, multivesicular bodies; nts,

nucleotides; PGK, phosphoglycerate kinase; PKm2, pyruvate kinase m2 isoform; RISC, RNA-induced silencing complex; ST, Shiga Toxin B chain; U, unit; VEGF, vascular endothelial growth factor.

Funding

R.C.L. and S.K.L. are funded by Agency for Science, Technology and Research (A*STAR), Singapore.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

References

- Mendicino, M., Bailey, A.M., Wonnacott, K., Puri, R.K. and Bauer, S.R. (2014) MSC-based product characterization for clinical trials: an FDA perspective. *Cell Stem Cell* **14**, 141–145 <https://doi.org/10.1016/j.stem.2014.01.013>
- Pittenger, M., Mackay, A., Beck, S., Jaiswal, R., Douglas, R., Mosca, J. et al. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143–147 <https://doi.org/10.1126/science.284.5411.143>
- Ferrand, J., Noel, D., Lehours, P., Prochazkova-Carlotti, M., Chambonnier, L., Menard, A. et al. (2011) Human bone marrow-derived stem cells acquire epithelial characteristics through fusion with gastrointestinal epithelial cells. *PLoS ONE* **6**, e19569 <https://doi.org/10.1371/journal.pone.0019569>
- Spees, J.L., Olson, S.D., Ylostalo, J., Lynch, P.J., Smith, J., Perry, A. et al. (2003) Differentiation, cell fusion and nuclear fusion during ex vivo repair of epithelium by human adult stem cells from bone marrow stroma. *Proc. Natl Acad. Sci. U.S.A.* **100**, 2397–2402 <https://doi.org/10.1073/pnas.0437997100>
- Vassilopoulos, G., Wang, P.R. and Russell, D.W. (2003) Transplanted bone marrow regenerates liver by cell fusion. *Nature* **422**, 901–904 <https://doi.org/10.1038/nature01539>
- Freyman, T., Polin, G., Osman, H., Crary, J., Lu, M., Cheng, L. et al. (2006) A quantitative, randomized study evaluating three methods of mesenchymal stem cell delivery following myocardial infarction. *Eur. Heart J.* **27**, 1114–1122 <https://doi.org/10.1093/eurheartj/ehi818>
- Toma, C., Pittenger, M.F., Cahill, K.S., Byrne, B.J. and Kessler, P.D. (2002) Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* **105**, 93–98 <https://doi.org/10.1161/hc0102.101442>
- Dai, W., Hale, S.L., Martin, B.J., Kuang, J.Q., Dow, J.S., Wold, L.E. et al. (2005) Allogeneic mesenchymal stem cell transplantation in postinfarcted rat myocardium: short- and long-term effects. *Circulation* **112**, 214–223 <https://doi.org/10.1161/CIRCULATIONAHA.104.527937>
- Iso, Y., Spees, J.L., Serrano, C., Bakondi, B., Pochampally, R., Song, Y.H. et al. (2007) Multipotent human stromal cells improve cardiac function after myocardial infarction in mice without long-term engraftment. *Biochem. Biophys. Res. Commun.* **354**, 700–706 <https://doi.org/10.1016/j.bbrc.2007.01.045>
- Gnecchi, M., He, H., Noiseux, N., Liang, O.D., Zhang, L., Morello, F. et al. (2006) Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *FASEB J.* **20**, 661–669 <https://doi.org/10.1096/fj.05-5211.com>
- da Silva Meirelles, L., Caplan, A.I. and Nardi, N.B. (2008) In search of the in vivo identity of mesenchymal stem cells. *Stem Cells* **26**, 2287–2299 <https://doi.org/10.1634/stemcells.2007-1122>
- Noiseux, N., Gnecchi, M., Lopez-Illasaca, M., Zhang, L., Solomon, S.D., Deb, A. et al. (2006) Mesenchymal stem cells overexpressing Akt dramatically repair infarcted myocardium and improve cardiac function despite infrequent cellular fusion or differentiation. *Mol. Ther.* **14**, 840–850 <https://doi.org/10.1016/j.ymthe.2006.05.016>
- Lee, R.H., Pulin, A.A., Seo, M.J., Kota, D.J., Ylostalo, J., Larson, B.L. et al. (2009) Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. *Cell Stem Cell* **5**, 54–63 <https://doi.org/10.1016/j.stem.2009.05.003>
- Caplan, A.I. and Dennis, J.E. (2006) Mesenchymal stem cells as trophic mediators. *J. Cell. Biochem.* **98**, 1076–1084 <https://doi.org/10.1002/jcb.20886>
- Kinnaird, T., Stabile, E., Burnett, M.S., Shou, M., Lee, C.W., Barr, S., Fuchs, S. and Epstein, S.E. (2004) Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation* **109**, 1543–1549 <https://doi.org/10.1161/01.CIR.0000124062.31102.57> PMID: 15023891
- Timmers, L., Lim, S.K., Arslan, F., Armstrong, J.S., Hoefler, I.E., Doevendans, P.A. et al. (2007) Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. *Stem Cell Res.* **1**, 129–137 <https://doi.org/10.1016/j.scr.2008.02.002>
- Giebel, B., Kordelas, L. and Börger, V. (2017) Clinical potential of mesenchymal stem/stromal cell-derived extracellular vesicles. *Stem Cell Investig.* **4**, 84 <https://doi.org/10.21037/sci.2017.09.06>
- Bruno, S., Grange, C., Deregibus, M.C., Calogero, R.A., Saviozzi, S., Collino, F. et al. (2009) Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. *J. Am. Soc. Nephrol.* **20**, 1053–1067 <https://doi.org/10.1681/ASN.2008070798>
- Lai, R.C., Arslan, F., Lee, M.M., Sze, N.S., Choo, A., Chen, T.S. et al. (2010) Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res.* **4**, 214–222 <https://doi.org/10.1016/j.scr.2009.12.003>
- They, C., Ostrowski, M. and Segura, E. (2009) Membrane vesicles as conveyors of immune responses. *Nat. Rev. Immunol.* **9**, 581–593 <https://doi.org/10.1038/nri2567>
- Fevrier, B. and Raposo, G. (2004) Exosomes: endosomal-derived vesicles shipping extracellular messages. *Curr. Opin. Cell Biol.* **16**, 415–421 <https://doi.org/10.1016/j.ceb.2004.06.003>
- Wubbolts, R., Leckie, R.S., Veenhuizen, P.T., Schwarzmann, G., Mobius, W., Hoernschemeyer, J. et al. (2003) Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *J. Biol. Chem.* **278**, 10963–10972 <https://doi.org/10.1074/jbc.M207550200>
- de Gassart, A., Geminard, C., Fevrier, B., Raposo, G. and Vidal, M. (2003) Lipid raft-associated protein sorting in exosomes. *Blood* **102**, 4336–4344 <https://doi.org/10.1182/blood-2003-03-0871>
- Simpson, R.J., Jensen, S.S. and Lim, J.W. (2008) Proteomic profiling of exosomes: current perspectives. *Proteomics* **8**, 4083–4099 <https://doi.org/10.1002/pmic.200800109>
- Pan, B.T. and Johnstone, R.M. (1983) Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell* **33**, 967–978 [https://doi.org/10.1016/0092-8674\(83\)90040-5](https://doi.org/10.1016/0092-8674(83)90040-5)

- 26 Dai, M., Yu, M., Zhang, Y. and Tian, W. (2017) Exosome-like vesicles derived from adipose tissue provide biochemical cues for adipose tissue regeneration. *Tissue Eng. A* **23**, 1221–1230 <https://doi.org/10.1089/ten.TEA.2017.0045>
- 27 Raposo, G., Nijman, H.W., Stoorvogel, W., Liejendekker, R., Harding, C.V., Melief, C.J. et al. (1996) B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.* **183**, 1161–1172 <https://doi.org/10.1084/jem.183.3.1161>
- 28 Bakhti, M., Winter, C. and Simons, M. (2011) Inhibition of myelin membrane sheath formation by oligodendrocyte-derived exosome-like vesicles. *J. Biol. Chem.* **286**, 787–796 <https://doi.org/10.1074/jbc.M110.190009>
- 29 Buck, A.H., Coakley, G., Simbari, F., McSorley, H.J., Quintana, J.F., Le Bihan, T. et al. (2014) Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity. *Nat. Commun.* **5**, 5488 <https://doi.org/10.1038/ncomms6488>
- 30 Vrijnsen, K.R., Sluijter, J.P., Schuchardt, M.W., van Balkom, B.W., Noort, W.A., Chamuleau, S.A. et al. (2010) Cardiomyocyte progenitor cell-derived exosomes stimulate migration of endothelial cells. *J. Cell. Mol. Med.* **14**, 1064–1070 <https://doi.org/10.1111/j.1582-4934.2010.01081.x>
- 31 Lachenal, G., Pernet-Gally, K., Chivet, M., Hemming, F.J., Belly, A., Bodon, G. et al. (2011) Release of exosomes from differentiated neurons and its regulation by synaptic glutamatergic activity. *Mol. Cell. Neurosci.* **46**, 409–418 <https://doi.org/10.1016/j.mcn.2010.11.004>
- 32 Pegtel, D.M., Cosmopoulos, K., Thorley-Lawson, D.A., van Eijndhoven, M.A., Hopmans, E.S., Lindenberg, J.L. et al. (2010) Functional delivery of viral miRNAs via exosomes. *Proc. Natl Acad. Sci. U.S.A.* **107**, 6328–6333 <https://doi.org/10.1073/pnas.0914843107>
- 33 Katakowski, M., Buller, B., Zheng, X., Lu, Y., Rogers, T., Osobamiro, O. et al. (2013) Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. *Cancer Lett.* **335**, 201–204 <https://doi.org/10.1016/j.canlet.2013.02.019>
- 34 Miyado, K., Yoshida, K., Yamagata, K., Sakakibara, K., Okabe, M., Wang, X. et al. (2008) The fusing ability of sperm is bestowed by CD9-containing vesicles released from eggs in mice. *Proc. Natl Acad. Sci. U.S.A.* **105**, 12921–12926 <https://doi.org/10.1073/pnas.0710608105>
- 35 Leblanc, P., Arellano-Anaya, Z.E., Bernard, E., Gally, L., Provansal, M., Lehmann, S. et al. (2017) Isolation of exosomes and microvesicles from cell culture systems to study prion transmission. *Methods Mol. Biol.* **1545**, 153–176 https://doi.org/10.1007/978-1-4939-6728-5_11
- 36 Teow, S.Y., Nordin, A.C., Ali, S.A. and Khoo, A.S. (2016) Exosomes in human immunodeficiency virus type I pathogenesis: threat or opportunity? *Adv. Virol.* **2016**, 9852494 <https://doi.org/10.1155/2016/9852494>
- 37 Ramakrishnaiah, V., Thumann, C., Fofana, I., Habersetzer, F., Pan, Q., de Ruiter, P.E. et al. (2013) Exosome-mediated transmission of hepatitis C virus between human hepatoma Huh7.5 cells. *Proc. Natl Acad. Sci. U.S.A.* **110**, 13109–13113 <https://doi.org/10.1073/pnas.1221899110>
- 38 Hao, S., Ye, Z., Li, F., Meng, Q., Qureshi, M., Yang, J. et al. (2006) Epigenetic transfer of metastatic activity by uptake of highly metastatic B16 melanoma cell-released exosomes. *Exp. Oncol.* **28**, 126–131 PMID:16837903
- 39 Richards, K.E., Zeleniak, A.E., Fishel, M.L., Wu, J., Littlepage, L.E. and Hill, R. (2017) Cancer-associated fibroblast exosomes regulate survival and proliferation of pancreatic cancer cells. *Oncogene* **36**, 1770–1778 <https://doi.org/10.1038/ncr.2016.353>
- 40 Chen, T.S., Lai, R.C., Lee, M.M., Choo, A.B., Lee, C.N. and Lim, S.K. (2010) Mesenchymal stem cell secretes microparticles enriched in pre-microRNAs. *Nucleic Acids Res.* **38**, 215–224 <https://doi.org/10.1093/nar/gkp857>
- 41 Li, T., Yan, Y., Wang, B., Qian, H., Zhang, X., Shen, L. et al. (2013) Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis. *Stem Cells Dev.* **22**, 845–854 <https://doi.org/10.1089/scd.2012.0395>
- 42 Yu, B., Shao, H., Su, C., Jiang, Y., Chen, X., Bai, L. et al. (2016) Exosomes derived from MSCs ameliorate retinal laser injury partially by inhibition of MCP-1. *Sci. Rep.* **6**, 34562 <https://doi.org/10.1038/srep34562>
- 43 Hu, G.W., Li, Q., Niu, X., Hu, B., Liu, J., Zhou, S.M. et al. (2015) Exosomes secreted by human-induced pluripotent stem cell-derived mesenchymal stem cells attenuate limb ischemia by promoting angiogenesis in mice. *Stem Cell Res. Ther.* **6**, 10 <https://doi.org/10.1186/s12916-015-0417-0>
- 44 Nakano, M., Nagaishi, K., Konari, N., Saito, Y., Chikenji, T., Mizue, Y. et al. (2016) Bone marrow-derived mesenchymal stem cells improve diabetes-induced cognitive impairment by exosome transfer into damaged neurons and astrocytes. *Sci. Rep.* **6**, 24805 <https://doi.org/10.1038/srep24805>
- 45 Drommschmidt, K., Serdar, M., Bendix, I., Herz, J., Bertling, F., Prager, S. et al. (2017) Mesenchymal stem cell-derived extracellular vesicles ameliorate inflammation-induced preterm brain injury. *Brain Behav. Immun.* **60**, 220–232 <https://doi.org/10.1016/j.bbi.2016.11.011>
- 46 Xin, H., Li, Y., Cui, Y., Yang, J.J., Zhang, Z.G. and Chopp, M. (2013) Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats. *J. Cereb. Blood Flow Metab.* **33**, 1711–1715 <https://doi.org/10.1038/jcbfm.2013.152>
- 47 Ono, M., Kosaka, N., Tominaga, N., Yoshioka, Y., Takeshita, F., Takahashi, R.U. et al. (2014) Exosomes from bone marrow mesenchymal stem cells contain a microRNA that promotes dormancy in metastatic breast cancer cells. *Sci. Signal.* **7**, ra63 <https://doi.org/10.1126/scisignal.2005231>
- 48 Tan, C.Y., Lai, R.C., Wong, W., Dan, Y.Y., Lim, S.K. and Ho, H.K. (2014) Mesenchymal stem cell-derived exosomes promote hepatic regeneration in drug-induced liver injury models. *Stem Cell Res. Ther.* **5**, 76 <https://doi.org/10.1186/s12916-014-0417-0>
- 49 Nakamura, Y., Miyaki, S., Ishitobi, H., Matsuyama, S., Nakasa, T., Kamei, N. et al. (2015) Mesenchymal-stem-cell-derived exosomes accelerate skeletal muscle regeneration. *FEBS Lett.* **589**, 1257–1265 <https://doi.org/10.1016/j.febslet.2015.03.031>
- 50 Zhang, J., Guan, J., Niu, X., Hu, G., Guo, S., Li, Q. et al. (2015) Exosomes released from human induced pluripotent stem cells-derived MSCs facilitate cutaneous wound healing by promoting collagen synthesis and angiogenesis. *J. Transl. Med.* **13**, 49 <https://doi.org/10.1186/s12967-015-0417-0>
- 51 Furuta, T., Miyaki, S., Ishitobi, H., Ogura, T., Kato, Y., Kamei, N. et al. (2016) Mesenchymal stem cell-derived exosomes promote fracture healing in a mouse model. *Stem Cells Transl. Med.* **5**, 1620–1630 <https://doi.org/10.5966/sctm.2015-0285>
- 52 Zhang, S., Chu, W., Lai, R., Hui, J., Lee, E., Lim, S. et al. (2016) Human mesenchymal stem cell-derived exosomes promote orderly cartilage regeneration in an immunocompetent rat osteochondral defect model. *Cytotherapy* **18**, S13 <https://doi.org/10.1016/j.jcyt.2016.03.035>
- 53 Zhang, S., Chuah, S.J., Lai, R.C., Hui, J.H.P., Lim, S.K. and Toh, W.S. (2018) MSC exosomes mediate cartilage repair by enhancing proliferation, attenuating apoptosis and modulating immune reactivity. *Biomaterials* **156**, 16–27 <https://doi.org/10.1016/j.biomaterials.2017.11.028>
- 54 Zhang, S., Chu, W., Lai, R., Lim, S., Hui, J. and Toh, W. (2016) Exosomes derived from human embryonic mesenchymal stem cells promote osteochondral regeneration. *Osteoarthritis Cartilage* **24**, 2135–2140 <https://doi.org/10.1016/j.joca.2016.06.022>
- 55 Fernandez, O., Izquierdo, G., Fernandez, V., Leyva, L., Reyes, V., Guerrero, M. et al. (2018) Adipose-derived mesenchymal stem cells (AdMSC) for the treatment of secondary-progressive multiple sclerosis: a triple blinded, placebo controlled, randomized phase I/II safety and feasibility study. *PLoS ONE* **13**, e0195891 <https://doi.org/10.1371/journal.pone.0195891>
- 56 Makhloogh, A., Shekarchian, S., Moghadasali, R., Einollahi, B., Dastgheib, M., Janbabaee, G. et al. (2018) Bone marrow-mesenchymal stromal cell infusion in patients with chronic kidney disease: a safety study with 18 months of follow-up. *Cytotherapy* **20**, 660–669 <https://doi.org/10.1016/j.jcyt.2018.02.368>

- 57 Bartolucci, J., Verdugo, F.J., Gonzalez, P.L., Larrea, R.E., Abarzua, E., Goset, C. et al. (2017) Safety and efficacy of the intravenous infusion of umbilical cord mesenchymal stem cells in patients with heart failure: a phase 1/2 randomized controlled trial (RIMECARD trial [randomized clinical trial of intravenous infusion umbilical cord mesenchymal stem cells on cardiopathy]). *Circ. Res.* **121**, 1192–1204 <https://doi.org/10.1161/CIRCRESAHA.117.310712>
- 58 Furlani, D., Ugurlucan, M., Ong, L., Bieback, K., Pittermann, E., Westien, I. et al. (2009) Is the intravascular administration of mesenchymal stem cells safe? Mesenchymal stem cells and intravital microscopy. *Microvasc. Res.* **77**, 370–376 <https://doi.org/10.1016/j.mvr.2009.02.001>
- 59 Jeong, J.O., Han, J.W., Kim, J.M., Cho, H.J., Park, C., Lee, N. et al. (2011) Malignant tumor formation after transplantation of short-term cultured bone marrow mesenchymal stem cells in experimental myocardial infarction and diabetic neuropathy. *Circ. Res.* **108**, 1340–1347 <https://doi.org/10.1161/CIRCRESAHA.110.239848>
- 60 Chang, M.G., Tung, L., Sekar, R.B., Chang, C.Y., Cysyk, J., Dong, P. et al. (2006) Proarrhythmic potential of mesenchymal stem cell transplantation revealed in an in vitro coculture model. *Circulation* **113**, 1832–1841 <https://doi.org/10.1161/CIRCULATIONAHA.105.593038>
- 61 Pak, H.N., Qayyum, M., Kim, D.T., Hamabe, A., Miyauchi, Y., Lill, M.C. et al. (2003) Mesenchymal stem cell injection induces cardiac nerve sprouting and increased tenascin expression in a Swine model of myocardial infarction. *J. Cardiovasc. Electrophysiol.* **14**, 841–848 <https://doi.org/10.1046/j.1540-8167.2003.03124.x>
- 62 Price, M.J., Chou, C.C., Frantzen, M., Miyamoto, T., Kar, S., Lee, S. et al. (2006) Intravenous mesenchymal stem cell therapy early after reperfused acute myocardial infarction improves left ventricular function and alters electrophysiologic properties. *Int. J. Cardiol.* **111**, 231–239 <https://doi.org/10.1016/j.ijcard.2005.07.036>
- 63 Breitbach, M., Bostani, T., Roell, W., Xia, Y., Dewald, O., Nygren, J.M. et al. (2007) Potential risks of bone marrow cell transplantation into infarcted hearts. *Blood* **110**, 1362–1369 <https://doi.org/10.1182/blood-2006-12-063412>
- 64 Chen, T.S., Arslan, F., Yin, Y., Tan, S.S., Lai, R.C., Choo, A.B. et al. (2011) Enabling a robust scalable manufacturing process for therapeutic exosomes through oncogenic immortalization of human ESC-derived MSCs. *J. Transl. Med.* **9**, 47 <https://doi.org/10.1186/1479-5876-9-47>
- 65 Lai, R.C., Tan, S.S., Yeo, R.W.Y., Choo, A.B.H., Reiner, A.T., Su, Y. et al. (2016) MSC secretes at least 3 EV types each with a unique permutation of membrane lipid, protein and RNA. *J. Extracell. Vesicles* **5**, 29828 <https://doi.org/10.3402/jev.v5.29828>
- 66 Tan, S.S., Yin, Y., Lee, T., Lai, R.C., Yeo, R.W., Zhang, B. et al. (2013) Therapeutic MSC exosomes are derived from lipid raft microdomains in the plasma membrane. *J. Extracell. Vesicles* **2**, 22614 <https://doi.org/10.3402/jev.v2i0.22614>
- 67 Smalheiser, N.R. (2007) Exosomal transfer of proteins and RNAs at synapses in the nervous system. *Biol. Direct* **2**, 35 <https://doi.org/10.1186/1745-6150-2-35>
- 68 Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J.J. and Lotvall, J.O. (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* **9**, 654–659 <https://doi.org/10.1038/ncb1596>
- 69 Pritchard, C.C., Cheng, H.H. and Tewari, M. (2012) MicroRNA profiling: approaches and considerations. *Nat. Rev. Genet.* **13**, 358–369 <https://doi.org/10.1038/nrg3198>
- 70 Baglio, S.R., Rooijers, K., Koppers-Lalic, D., Verweij, F.J., Pérez Lanzón, M., Zini, N. et al. (2015) Human bone marrow- and adipose-mesenchymal stem cells secrete exosomes enriched in distinctive miRNA and tRNA species. *Stem Cell Res. Ther.* **6**, 127 <https://doi.org/10.1186/s13287-015-0116-z>
- 71 Skog, J., Wurdinger, T., van Rijn, S., Meijer, D., Gainche, L., Sena-Estevés, M. et al. (2008) Glioblastoma microvesicles transport RNA and protein that promote tumor growth and provide diagnostic biomarkers. *Nat. Cell Biol.* **10**, 1470–1476 <https://doi.org/10.1038/ncb1800>
- 72 Kesimer, M., Scull, M., Brighton, B., DeMaria, G., Burns, K., O'Neal, W. et al. (2009) Characterization of exosome-like vesicles released from human tracheobronchial ciliated epithelium: a possible role in innate defense. *FASEB J.* **23**, 1858–1868 <https://doi.org/10.1096/fj.08-119131>
- 73 Kosaka, N., Iguchi, H., Yoshioka, Y., Takeshita, F., Matsuki, Y. and Ochiya, T. (2010) Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J. Biol. Chem.* **285**, 17442–17452 <https://doi.org/10.1074/jbc.M110.107821>
- 74 Nolte-'t Hoen, E.N.M., Buermans, H.P.J., Waasdorp, M., Stoorvogel, W., Wauben, M.H.M. and 't Hoen, P.A.C. (2012) Deep sequencing of RNA from immune cell-derived vesicles uncovers the selective incorporation of small non-coding RNA biotypes with potential regulatory functions. *Nucleic Acids Res.* **40**, 9272–9285 <https://doi.org/10.1093/nar/gks658>
- 75 Jenjaroenpun, P., Kremenska, Y., Nair, V.M., Kremenskoy, M., Joseph, B. and Kurochkin, I.V. (2013) Characterization of RNA in exosomes secreted by human breast cancer cell lines using next-generation sequencing. *Peer J.* **1**, e201 <https://doi.org/10.7717/peerj.201>
- 76 Enderle, D., Spiel, A., Coticchia, C.M., Berghoff, E., Mueller, R., Schlumpberger, M. et al. (2015) Characterization of RNA from exosomes and other extracellular vesicles isolated by a novel spin column-based method. *PLoS ONE* **10**, e0136133 <https://doi.org/10.1371/journal.pone.0136133>
- 77 Ota, T., Suzuki, Y., Nishikawa, T., Otsuki, T., Sugiyama, T., Irie, R. et al. (2003) Complete sequencing and characterization of 21,243 full-length human cDNAs. *Nat. Genet.* **36**, 40 <https://doi.org/10.1038/ng1285>
- 78 Lim, P.K., Bliss, S.A., Patel, S.A., Taborga, M., Dave, M.A., Gregory, L.A. et al. (2011) Gap junction-mediated import of microRNA from bone marrow stromal cells can elicit cell cycle quiescence in breast cancer cells. *Cancer Res.* **71**, 1550–1560 <https://doi.org/10.1158/0008-5472.CAN-10-2372>
- 79 Kim, Y. and Kim, V.N. (2012) MicroRNA factory: RISC assembly from precursor microRNAs. *Mol. Cell* **46**, 384–386 <https://doi.org/10.1016/j.molcel.2012.05.012>
- 80 Arroyo, J.D., Chevillet, J.R., Kroh, E.M., Ruf, I.K., Pritchard, C.C., Gibson, D.F. et al. (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc. Natl Acad. Sci. U.S.A.* **108**, 5003–5008 <https://doi.org/10.1073/pnas.1019055108>
- 81 Katsuda, T. and Ochiya, T. (2015) Molecular signatures of mesenchymal stem cell-derived extracellular vesicle-mediated tissue repair. *Stem Cell Res. Ther.* **6**, 212 <https://doi.org/10.1186/s13287-015-0214-y>
- 82 Liang, X., Zhang, L., Wang, S., Han, Q. and Zhao, R.C. (2016) Exosomes secreted by mesenchymal stem cells promote endothelial cell angiogenesis by transferring miR-125a. *J. Cell Sci.* **129**, 2182–2189 <https://doi.org/10.1242/jcs.170373>
- 83 Gong, M., Yu, B., Wang, J., Wang, Y., Liu, M., Paul, C. et al. (2017) Mesenchymal stem cells release exosomes that transfer miRNAs to endothelial cells and promote angiogenesis. *Oncotarget* **8**, 45200–45212 <https://doi.org/10.18632/oncotarget.16778>
- 84 Lee, C., Mitsialis, S.A., Aslam, M., Vitali, S.H., Vergadi, E., Konstantinou, G. et al. (2012) Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. *Circulation* **126**, 2601–2611 <https://doi.org/10.1161/CIRCULATIONAHA.112.114173>
- 85 Pakravan, K., Babashah, S., Sadeghizadeh, M., Mowla, S.J., Mossahebi-Mohammadi, M., Ataei, F. et al. (2017) MicroRNA-100 shuttled by mesenchymal stem cell-derived exosomes suppresses in vitro angiogenesis through modulating the mTOR/HIF-1 α /VEGF signaling axis in breast cancer cells. *Cell. Oncol.* **40**, 457–470 <https://doi.org/10.1007/s13402-017-0335-7>

- 86 Li, X., Liu, L., Yang, J., Yu, Y., Chai, J., Wang, L. et al. (2016) Exosome derived from human umbilical cord mesenchymal stem cell mediates miR-181c attenuating burn-induced excessive inflammation. *EBioMedicine* **8**, 72–82 <https://doi.org/10.1016/j.ebiom.2016.04.030>
- 87 Song, Y., Dou, H., Li, X., Zhao, X., Li, Y., Liu, D. et al. (2017) Exosomal miR-146a contributes to the enhanced therapeutic efficacy of interleukin-1 β -primed mesenchymal stem cells against sepsis. *Stem Cells* **35**, 1208–1221 <https://doi.org/10.1002/stem.2564>
- 88 Wang, X., Gu, H., Qin, D., Yang, L., Huang, W., Essandoh, K. et al. (2015) Exosomal miR-223 contributes to mesenchymal stem cell-elicited cardioprotection in polymicrobial sepsis. *Sci. Rep.* **5**, 13721 <https://doi.org/10.1038/srep13721>
- 89 Qu, Y., Zhang, Q., Cai, X., Li, F., Ma, Z., Xu, M. et al. (2017) Exosomes derived from miR-181-5p modified adipose-derived mesenchymal stem cells prevent liver fibrosis via autophagy activation. *J. Cell. Mol. Med.* **21**, 2491–2502 <https://doi.org/10.1111/jcmm.13170>
- 90 Lou, G., Yang, Y., Liu, F., Ye, B., Chen, Z., Zheng, M. et al. (2017) miR-122 modification enhances the therapeutic efficacy of adipose tissue-derived mesenchymal stem cells against liver fibrosis. *J. Cell. Mol. Med.* **21**, 2963–2973 <https://doi.org/10.1111/jcmm.13208>
- 91 Fang, S., Xu, C., Zhang, Y., Xue, C., Yang, C., Bi, H. et al. (2016) Umbilical cord-derived mesenchymal stem cell-derived exosomal microRNAs suppress myofibroblast differentiation by inhibiting the transforming growth factor- β /SMAD2 pathway during wound healing. *Stem Cells Transl. Med.* **5**, 1425–1439 <https://doi.org/10.5966/sctm.2015-0367>
- 92 Li, M., Zeringer, E., Barta, T., Schageman, J., Cheng, A. and Vlassov, A.V. (2014) Analysis of the RNA content of the exosomes derived from blood serum and urine and its potential as biomarkers. *Philos. Trans. R. Soc. B Biol. Sci.* **369**, 20130502 <https://doi.org/10.1098/rstb.2013.0502>
- 93 Fang, Z., Du, R., Edwards, A., Flemington, E.K. and Zhang, K. (2013) The sequence structures of human microRNA molecules and their implications. *PLoS ONE* **8**, e54215 <https://doi.org/10.1371/journal.pone.0054215>
- 94 Chevillet, J.R., Kang, Q., Ruf, I.K., Briggs, H.A., Vojtech, L.N., Hughes, S.M. et al. (2014) Quantitative and stoichiometric analysis of the microRNA content of exosomes. *Proc. Natl Acad. Sci. U.S.A.* **111**, 14888–14893 <https://doi.org/10.1073/pnas.1408301111>
- 95 Bissels, U., Wild, S., Tomiuk, S., Holste, A., Hafner, M., Tuschl, T. et al. (2009) Absolute quantification of microRNAs by using a universal reference. *RNA* **15**, 2375–2384 <https://doi.org/10.1261/ma.1754109>
- 96 Reich, S.J., Fosnot, J., Kuroki, A., Tang, W., Yang, X., Maguire, A.M. et al. (2003) Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. *Mol. Vis.* **9**, 210–216 PMID:12789138
- 97 Dorn, G., Patel, S., Wotherspoon, G., Hemmings-Mieszczak, M., Barclay, J., Natt, F.J.C. et al. (2004) siRNA relieves chronic neuropathic pain. *Nucleic Acids Res.* **32**, e49 <https://doi.org/10.1093/nar/gnh044>
- 98 Ganbold, T., Gerile, G., Xiao, H. and Baigude, H. (2017) Efficient in vivo siRNA delivery by stabilized d-peptide-based lipid nanoparticles. *RSC Adv.* **7**, 8823–8831 <https://doi.org/10.1039/C6RA25862J>
- 99 Anderson, J.D., Johansson, H.J., Graham, C.S., Vesterlund, M., Pham, M.T., Bramlett, C.S. et al. (2016) Comprehensive proteomic analysis of mesenchymal stem cell exosomes reveals modulation of angiogenesis via nuclear factor- κ B signaling. *Stem Cells* **34**, 601–613 <https://doi.org/10.1002/stem.2298>
- 100 Kim, H.-S., Choi, D.-Y., Yun, S.J., Choi, S.-M., Kang, J.W., Jung, J.W. et al. (2012) Proteomic analysis of microvesicles derived from human mesenchymal stem cells. *J. Proteome Res.* **11**, 839–849 <https://doi.org/10.1021/pr200682z>
- 101 Lai, R.C., Yeo, R.W., Tan, K.H. and Lim, S.K. (2013) Mesenchymal stem cell exosome ameliorates reperfusion injury through proteomic complementation. *Regen. Med.* **8**, 197–209 <https://doi.org/10.2217/rme.13.4>
- 102 Collino, F., Pomatto, M., Bruno, S., Lindoso, R.S., Tapparo, M., Sicheng, W. et al. (2017) Exosome and microvesicle-enriched fractions isolated from mesenchymal stem cells by gradient separation showed different molecular signatures and functions on renal tubular epithelial cells. *Stem Cell Rev.* **13**, 226–243 <https://doi.org/10.1007/s12015-016-9713-1>
- 103 Tan, S.S., Chen, T.S., Tan, K.H. and Lim, S.K. (2015) Chapter 2 — an overview of the proteomic and miRNA cargo in MSC-derived exosomes. In *Mesenchymal Stem Cell Derived Exosomes* (Dawn, B., Tang, Y., eds), pp. 21–36, Academic Press, Boston
- 104 Lai, R.C., Tan, S.S., Teh, B.J., Sze, S.K., Arslan, F., de Kleijn, D.P. et al. (2012) Proteolytic potential of the MSC exosome proteome: implications for an exosome-mediated delivery of therapeutic proteasome. *Int. J. Proteomics* **2012**, 971907 <https://doi.org/10.1155/2012/971907>
- 105 Lai, R.C., Arslan, F., Tan, S.S., Tan, B., Choo, A., Lee, M.M. et al. (2010) Derivation and characterization of human fetal MSCs: an alternative cell source for large-scale production of cardioprotective microparticles. *J. Mol. Cell. Cardiol.* **48**, 1215–1224 <https://doi.org/10.1016/j.yjmcc.2009.12.021>
- 106 Li, X., Arslan, F., Ren, Y., Adav, S.S., Poh, K.K., Sorokin, V. et al. (2012) Metabolic adaptation to a disruption in oxygen supply during myocardial ischemia and reperfusion is underpinned by temporal and quantitative changes in the cardiac proteome. *J. Proteome Res.* **11**, 2331–2346 <https://doi.org/10.1021/pr201025m>
- 107 Arslan, F., Lai, R.C., Smeets, M.B., Akeroyd, L., Choo, A., Aguor, E.N. et al. (2013) Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. *Stem Cell Res.* **10**, 301–312 <https://doi.org/10.1016/j.scr.2013.01.002>
- 108 Lai, R.C., Yeo, R.W., Tan, S.S., Zhang, B., Yin, Y., Sze, N.S. et al. (2012) Mesenchymal stem cell exosomes: the future MSC-based therapy?. In *Mesenchymal Stem Cell Therapy* (Chase, L.G. and Vemuri, M.C., eds), Humana Press
- 109 Beis, I. and Newsholme, E.A. (1975) The contents of adenine nucleotides, phosphagens and some glycolytic intermediates in resting muscles from vertebrates and invertebrates. *Biochem. J.* **152**, 23–32 <https://doi.org/10.1042/bj1520023>
- 110 Del Monte, U. (2009) Does the cell number 109 still really fit one gram of tumor tissue? *Cell Cycle* **8**, 505–506 <https://doi.org/10.4161/cc.8.3.7608>
- 111 Flamholz, A., Phillips, R. and Milo, R. (2014) The quantified cell. *Mol. Biol. Cell* **25**, 3497–3500 <https://doi.org/10.1091/mbc.e14-09-1347>