

Exosome: A Novel and Safer Therapeutic Refinement of Mesenchymal Stem Cell

Ronne Wee Yeh Yeo¹, Ruenn Chai Lai¹, Kok Hian Tan² and Sai Kiang Lim^{1,3*}

¹ Institute of Medical Biology, Agency for Science, Technology and Research, Singapore

² Department of Maternal Fetal Medicine, KK Women's and Children's Hospital, Singapore

³ Department of Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

*Corresponding author(s) E-mail: saikiang.lim@imb.a-star.edu.sg

Received 14 November 2013; Accepted

© 2013 The Author(s). Licensee InTech. This is an open access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/3.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Mesenchymal stem cell (MSC) has just been approved as the first “off-the-shelf” stem cell pharmaceutical drug with an anticipation of more approvals following completion of numerous rigorous clinical trials. Despite this progress, the rationale for MSC therapeutic efficacy remains tenuous and is increasingly rationalized on a secretion rather than differentiation mechanism. Recent studies identifying exosome as the secreted agent mediating MSC therapeutic efficacy could potentially reduce a cell-based drug to a safer biologic-based alternative. Here we review the development of MSC exosome as a potential first-in-class therapeutic, and the unique challenges in the manufacture and regulatory oversight of this new class of therapeutics.

Keywords Exosomes, Mesenchymal Stem Cell, Myocardial Ischemia / Reperfusion Injury, Purification

1. Introduction

1.1 Mesenchymal stem cells as a therapeutic agent

MSCs are currently the most clinically evaluated stem cells. The ClinicalTrials.gov database lists more than 300 MSC trials to treat a wide range of pathological conditions

(<http://www.clinicaltrials.gov/>, accessed March 2013). Many of these trials were predicated on the efficacy of MSC against a wide range of diseases in animal models. The therapeutic efficacy of transplanted MSCs was initially attributed to their homing and engraftment in injured tissues, and subsequent differentiation to repair and replace damaged tissues. However, studies in animal models and patients indicated that <1% of transplanted MSCs localize to the target tissue with most becoming trapped in the liver, spleen and lungs [1]. Furthermore, evidence for differentiation of transplanted MSCs at the site of injury could not rule out the possibility of fusion with endogenous cells [2-4]. In fact, the efficacy of MSC transplantation in treating diseases in animal models and patients has been increasingly observed to be independent of engraftment or differentiation [5-9]. Consequently, MSC secretion is now implicated as the primary mediator of MSC-based therapy.

The refinement of MSC therapy from a cell-to secretion-based therapy offers several advantages as it essentially translates the therapeutic agent from a living to non-living agent and obliterates the arduous task of preserving cell viability and function during manufacture, storage and delivery to patient. As such, cellular secretions are more amenable to development as an “off-the-shelf” therapeutic that can be delivered to patients in a timely manner. They also mitigate the safety risks inherent in administering

large, viable cells such as the risk of occlusion in microvasculature or unregulated growth. For example, intra-arterial administration of MSCs causes a high 25-40% incidence of pulmonary embolism and death [10]. MSC transplantation has also been associated with adverse cellular growth such as cardiac sympathetic nerve sprouting and consequently hyper-innervation [11], or ossification and/or calcification in cryo-infarcted hearts [12]. Therefore, the benefits of secretion-based therapy coupled with the risks of cell transplantations have spurred recent research interest in MSC secretion.

Much of the initial attention on MSC secretion has centered on small molecules such as growth factors, chemokines and cytokines [13]. Although many candidates for mediating the efficacy of MSC have been proposed, none could sufficiently account for the efficacy of MSC against the diverse range of pathological conditions. For example, several cytokines, chemokines and enzymes shown to be secreted by MSCs have been proposed as the secreted factor mediating immunotherapeutic efficacy of MSCs [14]. They include interferon- γ (IFN γ) [15-17], a downstream IFN γ -inducible gene indoleamine 2,3-dioxygenase (IDO) [18] and inducible nitric-oxide synthase (iNOS) [19]. Both IFN γ and IDO were eliminated as candidates when the immunomodulatory activity of MSCs was subsequently found not to be ameliorated by a lack of IDO production caused by either defective IFN γ receptor 1 or IDO inhibitors [20]. Also, murine MSCs unlike human MSCs do not have IDO activity but could exert similar immunomodulatory effects [21, 22]. In the case of inducible nitric-oxide synthase (iNOS) which appear to be important for the inhibition of T-cell proliferation by mouse MSCs, it was deemed not important in human MSCs as they expressed low level of iNOS [14]. MSCs are also known to secrete many generic immuno-modulating factors such as transforming growth factor (TGF)- β 1, hepatocyte growth factor (HGF), heme oxygenase 1, IL-6, prostaglandin E2 (PGE2), and HLA-G5 [23-34] and it is unlikely that any one of these factors could sufficiently mediate the immunotherapeutic efficacy of MSCs.

Efforts to identify the therapeutic agent in MSC secretion have recently shifted from the small molecules to relatively large secreted lipid membrane vesicles known collectively as extracellular vesicles (EVs) and these EVs were shown to be as therapeutically efficacious as MSCs [35-37].

2. Exosome, an extracellular vesicle (EV)

EVs consist of several classes of secreted vesicles such as exosomes, microvesicles, ectosomes, membrane particles, exosome-like vesicles or apoptotic bodies [38]. However, these different classes remain poorly defined and terms "exosomes", "microvesicles", "microparticles" are being used interchangeably.

Generally, EVs could be formed by either inward budding of endolysosomal vesicles followed by exocyto-

sis e.g. exosomes, or shedding from the plasma membrane e.g. microvesicles. Of the EVs characterized to date, exosomes remain the best characterized and are the only EVs known to be formed by the invagination of endolysosomal vesicles. Unlike other EVs, exosomes are defined as having a diameter of 40-100 nm, a flotation density in sucrose of 1.1-1.18 g/ml, and bilipid membrane enriched in lipid rafts of cholesterol, sphingomyelin, and ceramide [39, 40]. The presence of exposed phosphatidylserine on exosome membrane is controversial and remains to be determined [41-44].

Many cell types such as B cells [45], dendritic cells [46], mast cells [47], T cells [48], platelets [44], Schwann cells [49], tumor cells [50], mesenchymal stem cells [35], human embryonic kidney cells [51], various cancer cell lines [52] and sperms [53] are known to secrete exosomes. Consistent with this diverse range of cell types, exosomes are found in as many physiological fluids including bronchial lavage fluid [54], human urine [55, 56] and human blood [56]. Together, these indicate that exosome secretion is a conserved cellular function.

Exosomes from different cellular sources share an evolutionary conserved set of protein molecules including tetraspanins (CD81, CD63, CD9), Alix and Tsg101 but they also carry proteins and RNAs unique to their cell source and the pathophysiological states of the cell source [57]. For example, exosomes from reticulocytes, unlike those from lymphocytes and dendritic cells, are rich in transferrin receptors that had to be disposed of when reticulocytes mature [45, 58, 59] while exosomes from tumor cells carry tumor antigens [50, 60-62] or tumor-specific microRNAs [63]. The cargo composition of exosomes is also modulated by its microenvironment. Exosomes secreted by tumor cells during hypoxia carry proteins that facilitate angiogenesis and metastasis [64]. The list of proteins and RNAs reported to be present in exosomes are freely accessible at Exocarta (<http://www.exocarta.org>) or Vesiclepedia (<http://www.microvesicles.org>).

The biogenesis of exosomes in the endosomal pathway involves the invagination of the endosomal membrane to form intraluminal vesicles (ILVs) resulting in the formation of a multivesicular body (MVB). The ILVs will either be degraded if the MVB fuses with a lysosome or released as exosomes if the MVB fuses with the plasma membrane [65]. The process by which cellular proteins and RNAs is targeted to endosomes and subsequently exosomes has not been elucidated. Although ESCRT (endosomal sorting complex required for transport) has been implicated [66], some proteins have been shown to be targeted to exosomes in an ESCRT-independent manner via higher-order oligomerization or a ceramide-dependent but ESCRT-independent process [67, 68]. Notwithstanding this, it is obvious that the biogenesis of exosomes requires cellular expenditure of energy and resources, implying a functional importance for exosomes.

Despite its discovery about four decades ago [69], research interest in exosomes was fueled only recently when exosomes were found to be secreted by different cell types and participate in many important cellular activities. For example, exosomes are secreted by dendritic cells pulsed with tumor peptides for tumor suppression [70], by neurons for neurotransmission [71, 72], by oligodendrocytes for myelin membrane biogenesis [73], and by cardiomyocyte progenitor cells for migration of endothelial cells [74]. Exosomes from mesenchymal stem cells were even found to be therapeutic and could reduce myocardial ischemia/reperfusion injury [35] or acute tubular injury [36].

Unfortunately, exosomes have also been implicated in a wide spectrum of diseases from cancers and degenerative diseases to infectious diseases. Exosomes secreted by tumor cells have been shown to be capable of enhancing tumor or metastatic phenotype [75-77]. They are also implicated in the formation of disease-associated protein aggregates such as alpha-synuclein aggregates in Parkinson's disease [2, 78] and amyloid beta protein aggregates (A β) in Alzheimer disease [79]. It was also reported that during infection with diverse infectious agents such as scrapie, viruses and bacteria, cells secrete exosomes containing the infectious agent or its products and thus providing potential route for disseminating the infection [49, 70, 80-84].

As a consequence of the close association between the composition of exosomes and physiological or pathological states of the secreting cells, exosomes are good sentinels of cellular health and pathology, and have become an attractive source of disease biomarkers (reviewed [85]).

3. Exosomes as a therapeutic agent

The therapeutic potential of exosomes was first described in MSC exosomes when it was observed that the exosomes were cardioprotective in a murine model of acute myocardial ischemia/reperfusion injury [35]. This observation provided a rationale for an increasing MSC enigma.

The cardioprotective potential of MSCs have been widely reported; they reduce infarct size, improve left ventricular ejection fraction, and increase vascular density and myocardial perfusion in animal models of AMI [86-90]. Although this protection has always been rationalized on the potential of MSCs to differentiate and replace damaged cardiac tissues, significant engraftment and differentiation of MSCs into cardiomyocytes were seldom observed even when there were functional improvements [1-4]. In general, it was observed that the therapeutic efficacy of MSCs was not dependent on their physical proximity to the tissue [37-41] leading to the proposal that this therapeutic efficacy was mediated by secreted factors [42].

Timmers et al. first reported in 2008 that MSC secretion mediated the cardioprotection of MSC. They observed that after sterile filtration with a 0.2 μ m filter, culture medium

conditioned by human ESC-derived MSCs could reduce infarct size in pig and mouse models of acute myocardial ischemia and reperfusion injury [91]. This conditioned medium (CM) could also improve cardiac function in a pig model of myocardial ischemia by stimulating angiogenesis and preventing adverse myocardium [92]. Size fractionation of the CM subsequently revealed that the therapeutic activity resided in the 100 to 220 nm fraction. This fraction when purified by size-exclusion HPLC was found to be enriched in lipid membrane vesicles that fulfilled the defining criteria of exosomes i.e. 40-100 nm, a flotation density in sucrose of 1.1-1.18 g/ml, lipid membrane enriched in lipid rafts of cholesterol, sphingomyelin, and ceramide, and a conserved set of protein molecules, namely the tetraspanins (CD81, CD63, CD9), Alix and Tsg101 [39, 40]. This fraction of exosomes was able to reduce the infarct size in a mouse model of acute myocardial ischemia and reperfusion injury [35]. Although our lab was the first to describe the secretion and therapeutic potential of exosomes from MSCs, Bruno et al. preceded us in describing MSC secretion of lipid membrane vesicles which they termed "microvesicles" and the amelioration of glycerol-induced acute kidney injury by these vesicles [36]. As these microvesicles were not evaluated for exosome-associated properties and had an estimated diameter of 180 nm versus the 55-65 nm hydrodynamic radius [35] for the exosomes that we isolated, we postulated that these two populations of EVs are different.

In the last two years, MSC exosomes have been found to be efficacious in an increasing number of animal models for human diseases such as cerebral ischemia [93], liver fibrosis [94], hypoxic pulmonary hypertension [95], and acute kidney injury [96, 97]. Recently, MSC exosomes were administered to a treatment-resistant grade IV acute GVHD patient. Her symptoms were dramatically alleviated and she remained stable for five months [98].

4. Translating MSC exosome into a cardiac pharmaceutical drug

Acute myocardial infarction (AMI) or heart attack causes ~7.2 million deaths globally each year and inflicts a heavy socioeconomic burden [99]. While advances in reperfusion therapy to restore blood flow and oxygenation to ischemic heart tissues have significantly improved AMI survival rate [100], 65% of AMI survivors go on to develop fatal heart failure within 5 years [101]. This progression to heart failure is complex but is largely determined by the final size of infarcted myocardium in surviving AMI patients. Although early reperfusion of ischemic myocardium salvages much of the tissue at risk, reperfusion of severely ischemic tissue paradoxically causes lethal injury, known as ischemia/reperfusion (I/R) injury and is estimated to contribute up to 50% of the final infarct size in reperfused AMI patients [102]. To date, I/R injury has been intractable to pharmaceutical interventions [103]. The efficacy of MSC exosomes in reducing reperfusion injury and long term preservation

of cardiac function and geometry in animal models of AMI [35, 91, 92, 104, 105] therefore provides a compelling rationale for its translation into a pharmaceutical drug to treat I/R injury. If successful, exosome will be a “first-in-class”. This, in itself will present unique challenges in the manufacture and regulatory oversight.

5. MSC exosome-mediated efficacy against reperfusion injury

As the terms “exosomes”, “microvesicles”, “microparticles” are presently being used interchangeably in the literature, the term “MSC exosome” in this section shall be used in a similar context to include all extracellular vesicles produced by MSCs. MSC exosomes, like their prodigious cell source, are reportedly efficacious in an equally wide range of diseases. However, it remains to be determined if a single class of exosomes or a single molecular mechanism is responsible for the therapeutic efficacy against these different diseases. We have previously proposed that MSC exosomes ameliorate reperfusion injury through a proteomic complementation [106].

During acute myocardial ischemia, rapid reperfusion to re-oxygenate the tissues halts ischemia-induced cellular damages and restores cardiac contractibility but delayed reperfusion after 40-60 minutes of ischemia cannot restore cardiac contractibility [107-111]. This failure to restore cardiac contractibility in severely ischemic myocardial tissues has been partially attributed to I/R injury.

During ischemia, the lack of oxygen inhibits oxidative phosphorylation and causes ATP production to collapse. This is particularly detrimental to contractile function of cardiomyocytes which have the highest basal ATP consumption rate in a resting body. Cardiomyocytes derive 95% of its ATP from the highly efficient oxygen-dependent coupling of fatty acid β -oxidation and oxidative phosphorylation [112]. During ischemia, the lack of oxygen disrupts this ATP production and aerobic glycolysis, leaving anaerobic glycolysis as the major source of ATP production. Consequently, the cells become ATP deficient and ATP-dependent cellular activities such as protein synthesis and turnover, gene transcription, ion pumps or transporters have to be curtailed. Over time, this escalates to a loss of cellular homeostasis such as intracellular acidification, imbalance of ions such as sodium, potassium and calcium, and increased intracellular osmotic pressure through the accumulation of metabolic products such as ADP, inorganic phosphates, reduced coenzymes (e.g. NADH and FADH) and lactic acid, and eventually initiation of apoptosis [108-113]. Therefore, restoring ATP production and delaying initiation of apoptosis are critical therapeutic targets in reperfusion therapy.

In less ischemic tissues, reperfusion re-oxygenates and re-energizes the mitochondria to generate ATP and restores cellular activities. However, in the severely ischemic tissues where the cellular biochemistry is highly deranged, re-

oxygenation exacerbates mitochondrial damage and the perturbed ion homeostasis to cause I/R injury. Reperfusion of severely ischemic cells not only fails to restore oxidative phosphorylation but causes further damages such as opening of the mitochondrial permeability transition pore (MPTP) to generate and release reactive oxygen species (ROS), and aggravation of ionic imbalances leading to sarcolemmal disruption and hypercontracture [112, 114-116]. These ultimately result in initiation of apoptosis and an acute inflammatory response, both of which contribute to infarct size [87-90].

Our recent analysis of the cardiac proteome after ischemia and then reperfusion revealed that the dominant determinants of I/R injury, ATP production and apoptosis, are underpinned by proteomic changes that persist for at least 60 minutes post-reperfusion [117]. Therefore, therapeutic strategies targeting these determinants of I/R injury will have to rapidly overcome these proteomic alterations in order to alleviate the ATP deficit and vulnerability to apoptosis. We had proposed that protein complementation by MSC exosome could provide a direct intervention to overcome these proteomic alterations and elicit a timely cellular response that is critical for the survival of the ATP-deficient and pro-apoptotic reperfused cells [106].

MSC exosomes carry a full enzyme complement for the ATP-generating phase of glycolysis and also CD73, an adenosine-generating extracellular enzyme [118]. We had demonstrated that these enzymes were active, and MSC exosomes could increase ATP production and adenosine-mediated survival kinase signaling in cells [118] and a mouse model of I/R [105] (Figure 1). As I/R injury is complex and causes extensive biochemical derangements as discussed earlier, ameliorating ATP deficit and circumventing initiation of apoptosis essentially provide a short time window of opportunity for the cells to rectify other molecular derangements.

The remarkable efficacy of MSC exosome in reducing I/R injury in animal models and its large diverse proteome suggest that MSC exosome must have biochemical activities beyond the glycolytic enzymes and CD73 to rectify the other molecular derangements in I/R injury. Some of these activities include functionally active 20S proteasomes capable of reducing denatured protein aggregates in reperfused hearts of mouse models [119] and the inhibition of complement-mediated lysis by exosome-associated CD59 [118]. We hypothesize that by rapidly correcting a critical ATP deficit and delaying initiation of apoptosis, the reperfused cells would have sufficient ATP to support the diverse biochemical potential of MSC exosomes and/or the endogenous repair mechanisms in the cells to rectify the other molecular derangements caused by I/R injury [106].

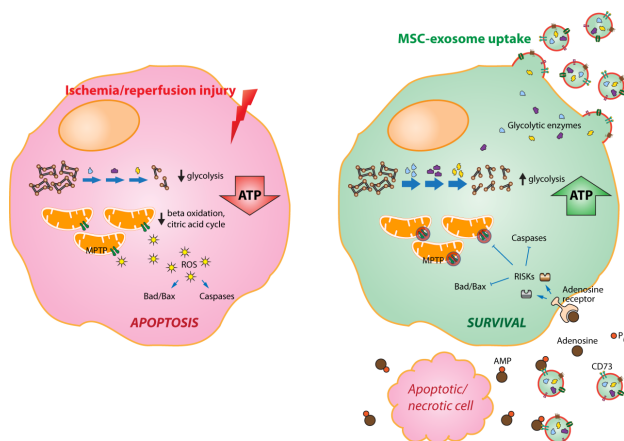


Figure 1. MSC exosomes ameliorate ischemia/ reperfusion injury by correcting ATP deficit and circumventing a pro-apoptotic proteome. In a severely ischemic myocardium, ATP-generating processes such as aerobic glycolysis, β -oxidation and citric acid cycle are curtailed, leading to a loss of cellular homeostasis. Reperfusion further exacerbates this injury by opening the mitochondrial permeability transition pore (MPTP) to release reactive oxygen species (ROS), resulting in the initiation of apoptosis. Exosomes ameliorate the ATP deficit by provide a full complement of glycolytic enzymes to enhance glycolytic flux. In addition, AMP derived from the degradation of ATP and ADP from injured or dying cells is hydrolyzed into adenosine by CD73 present on exosome surfaces. Adenosine in turn binds to adenosine receptors to activate reperfusion injury salvage kinase (RISK) pathways that mediate cell survival.

The effectiveness of MSC exosomes in ameliorating I/R injury could be partially attributed to the use of enzymes as therapeutic agents. By using enzymes, MSC exosomes could exert an amplified catalytic effect that is calibrated to their microenvironment (e.g., substrate concentration or pH). In healthy tissues where the microenvironment for most enzymes is likely to be in homeostatic equilibrium, activity of administered enzymes will be modulated by regulatory feedback mechanisms to maintain this equilibrium. During injury when homeostasis is disrupted, enzyme activity will be modulated in proportion to the magnitude of loss in homeostasis which is, in turn proportional to injury severity until equilibrium is restored or injury is resolved. This potential to sense and respond accordingly to restore equilibrium mitigates the risk of over- or under-dosing. The use of enzymes as therapeutics is traditionally limited by the susceptibility to degradation and by the lack of a vehicle to transfer proteins intracellularly. Their encapsulation in exosomes circumvents many of these limitations by protecting them against degradation to enhance their bioavailability, homing and enabling internalization by membrane fusion or endocytosis (reviewed [120]). In fact, the integrity of the exosome membrane is key to its efficacy in reducing I/R injury. When membrane integrity is compromised, exosomes lose their efficacy [105]. Interestingly, the efficiency of exosome uptake has been correlated to intracellular and microenvironmental acidity [121]. This provides a mechanism for exosome homing to ischemic tissues whereby MSC exosomes are preferentially endocytosed by ischemic cardiomyocytes which reportedly have low intracellular pH [122].

6. MSC exosome-mediated efficacy against other pathologies

Unlike reperfusion injury, the proposed mechanism of MSC exosome-mediated efficacy against other diseases have centered on either the RNA components of MSC exosomes or the RNA machinery of the target cells.

In glycerol-, gentamicin-, and cisplatin-induced acute kidney injury, MSC exosomes reportedly shuttle mRNA to tubular epithelial cells to promote proliferation and survival [36, 96, 97]. In a rat model of middle cerebral artery occlusion, it was demonstrated that MSC exosomes transferred miR-133b to neurons and astrocytes to promote neurite outgrowth and functional recovery after stroke [93].

On the other hand, MSC exosomes instead of delivering RNA were recently shown to suppress induction of the proliferative miR-17 microRNA superfamily but induce expression of antiproliferative miR-204, and prevent pulmonary vascular remodeling in a murine model of hypoxic pulmonary hypertension [95].

Together, the studies of MSC exosomes in animal models of diseases have implicated both proteins and RNAs in mediating their therapeutic efficacy against different diseases. Such versatility in the mode of action and disease targets is possible only because of the large payload that exosomes could carry and this clearly differentiates exosomes from traditional biologics.

7. MSC exosomes as a pharmaceutical drug

The main considerations in developing MSC exosomes as a pharmaceutical drug are the manufacturing path and regulatory oversight.

The key factor in the manufacture of MSC exosomes is the cell source. The ideal cell source would be a reproducibly high exosome-yielding cell with an infinite expansion capacity. We have observed that exosome production was inversely correlated with the age of the donor tissue with the most prolific producer being MSCs derived from hESCs followed by fetal tissues, umbilical cord and adult bone marrow (Chen et al, 2013). hESC-MSCs are also superior to those derived from other tissues in having a rapid doubling time of about 72 hours and being proliferative for at least 20 passages at a 1:4 split [123]. However, unlike other MSCs, hESC-MSCs are encumbered by the same social, legislative and ethical controversies surrounding hESCs. Although these controversies have abated, complex patent protection and legislation have become increasingly intractable barriers to translation [124, 125]. Also, the large proliferative capacity of hESC-MSCs is finite and would require costly and time-consuming repeated derivation and validation to sustain a manufacturing process. To overcome this issue, we immortalized hESC-MSCs by over-expressing *MYC* and demonstrated that despite a compromised differentiation potential, the immortalized cells continued to secrete

cardioprotective exosomes [126]. Notably, MYC protein was not detectable in the exosomes. Also, MYC RNA is not likely to be present in MSC exosomes as the RNAs in MSC exosome are primarily less than 300 nt [127].

Another key manufacturing consideration is the purification of exosomes. Exosomes are conventionally purified by ultracentrifugation, ultra-filtration and gel filtration [128-131]. While these techniques result in exosome enrichment, the preparation is usually contaminated with protein aggregates and other cell debris. Additionally, this method is time-consuming, requires expensive specialized equipment or has poor scalability. We have previously reported that the purity of the exosome preparation could be greatly enhanced by size exclusion high performance liquid chromatography (HPLC) [35, 104]. However, this method requires expensive equipment, and has poor scalability and product yield. While immuno-affinity chromatography [132, 133] is highly scalable and could potentially enhance the purity of the exosome preparations, none of the known exosome-associated antigens are exclusive to exosomes and immuno-affinity-based isolation protocols could still purify protein complexes and other EVs. In addition, the non-physiological salt or pH concentration needed to extract exosomes from the immuno-affinity column could affect the biological activity of the exosomes. In short, to date there is no ideal, scalable and cost effective method for the purification of exosomes.

The lack of defining markers for exosomes also poses a challenge to the development of an identity for exosomes as therapeutic agents. Exosomes are typically identified as particles of 100-200 nm in diameter, a density of 1.10-1.18 g/ml in sucrose and marked by tetraspanins CD9, CD81 or CD63) on their surfaces. These parameters are either too generic or too cumbersome for routine identification assay. Another challenge is a standardized unit of quantity to measure exosomes. The current use of protein concentration is ambiguous as it is highly dependent on the purity of the preparation. This challenge has been somewhat mitigated by the recent advent of particle analysis technologies such as Nanosight's Particle Tracking Analysis and Izon qNano's Tunable Resistive Pulse Sensing which quantify exosomes as particle numbers. Additionally, these technologies could profile size distribution of the particles which is useful in assessing of the level of contaminating EV populations (e.g. exosomes, microvesicles, apoptotic bodies) in the exosome preparation. However, particle count and size do not differentiate EV from non-EV particulates such as protein aggregates. A more specific quantification of exosome would be to assay for exosome-associated membrane-bound antigen (e.g. tetraspanins CD9, CD81 or CD63) by ELISA. However, there are no definitive exosome markers, and most exosome-associated markers are also found on other EVs. Essentially, there is an urgent need for an unambiguous assay to identify and quantify exosomes.

A third and probably most important challenge in developing exosomes as a pharmaceutical drug is the development of an in vitro surrogate assay for in vivo therapeutic potency. In the development of MSC exosome for the treatment of I/R injury, we envisage that such an assay is likely to involve the measurement of enzyme activity in intact exosomes as our observations thus far have indicated that therapeutic efficacy of MSC exosomes is dependent on enzyme activity and intact exosome membrane.

8. Regulatory oversight

The use of exosomes as a pharmaceutical product presently has no precedent and therefore has no definitive product "class" guidance within regulatory agencies such as the FDA and EMEA to instruct compliance. Nonetheless, the over-arching guidance could be easily surmised from the universal principle of safety, quality and efficacy. MSC exosome derives from a cell source that has been extensively tested in clinical trials for numerous indications. In general, administration of autologous and allogeneic MSCs has been found to be safe in humans and allogeneic MSCs are well tolerated without overt immune rejection. By extrapolation, MSC exosome which is essentially a minor constituent of MSC would be expected to be equally safe and immunologically tolerated. Consistent with the immune tolerance of MSC exosomes, proteomic analysis of MSC exosome revealed the absence of MHC and co-stimulatory molecules e.g. CD80 and CD86 [118, 119]. Finally, MSC exosomes have been demonstrated to be efficacious in clinically relevant animal models [35, 91, 92, 104, 105]. Together, experimental and pre-clinical data support MSC exosome as a safe and efficacious therapeutic agent that could be manufactured reproducibly, and provides a compelling rationale for further clinical development.

9. Conclusion

The efficacy of MSC against diverse disease indications is increasingly being attributed to its exosomes. This efficacy, which is prodigious for a single therapeutic entity, is not unreasonable particularly in view of its diverse cargo of proteins and RNA. In fact, this diverse cargo load provides a distinct advantage over a single molecule drug in enabling a simultaneous targeting of multiple disease processes through a multitude of mechanisms of action, an essential therapeutic strategy for complex injury or disease. Interestingly, many of the mechanisms of action are mediated by proteins and RNAs that are generally expressed in a wide variety of cell types, and that target generic housekeeping processes. These suggest that MSC exosomes exert their efficacy by restoring basic housekeeping activities in the injured cell and its microenvironment to facilitate endogenous repair and regeneration, and this therapeutic strategy resonates well with the role of MSC as a stromal support cell.

The identification of exosomes as the main agent mediating the therapeutic efficacy of MSCs provides a rationale for refining MSC-based therapy from a cellular to a non-cellular one. Although an exosome-based therapy could potentially reduce the complexities in the manufacturing and use of a cell-based product, the clinical development of exosome as a “first in class” drug presents unique but highly tractable manufacturing and regulatory challenges.

10. References

- [1] Phinney DG, Prockop DJ (2007) Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. *Stem Cells* 25: 2896-2902
- [2] Ferrand J, Noel D, Lehours P, et al. (2011) Human bone marrow-derived stem cells acquire epithelial characteristics through fusion with gastrointestinal epithelial cells. *PLoS One* 6: e19569
- [3] Spees JL, Olson SD, Ylostalo J, 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
- [4] Vassilopoulos G, Wang PR, Russell DW (2003) Transplanted bone marrow regenerates liver by cell fusion. *Nature* 422: 901-904
- [5] Prockop DJ (2007) "Stemness" does not explain the repair of many tissues by mesenchymal stem/multipotent stromal cells (MSCs). *Clinical pharmacology and therapeutics* 82: 241-243
- [6] da Silva Meirelles L, Caplan AI, Nardi NB (2008) In search of the in vivo identity of mesenchymal stem cells. *Stem Cells* 26: 2287-2299
- [7] Dai W, Hale SL, Martin BJ, et al. (2005) Allogeneic mesenchymal stem cell transplantation in postinfarcted rat myocardium: short-and long-term effects. *Circulation* 112: 214-223
- [8] Noiseux N, Gnechi M, Lopez-Illasaca M, 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
- [9] Iso Y, Spees JL, Serrano C, 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
- [10] Furlani D, Ugurlucan M, Ong L, et al. (2009) Is the intravascular administration of mesenchymal stem cells safe? Mesenchymal stem cells and intravital microscopy. *Microvasc Res* 77: 370-376
- [11] Pak HN, Qayyum M, Kim DT, 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
- [12] Breitbach M, Bostani T, Roell W, et al. (2007) Potential risks of bone marrow cell transplantation into infarcted hearts. *Blood* 110: 1362-1369
- [13] Caplan AI, Dennis JE (2006) Mesenchymal stem cells as trophic mediators. *J Cell Biochem* 98: 1076-1084
- [14] Ghannam S, Bouffi C, Djouad F, Jorgensen C, Noel D (2010) Immunosuppression by mesenchymal stem cells: mechanisms and clinical applications. *Stem Cell Research & Therapy* 1: 2
- [15] Rasmusson I, Ringden O, Sundberg B, Le Blanc K (2005) Mesenchymal stem cells inhibit lymphocyte proliferation by mitogens and alloantigens by different mechanisms. *Exp Cell Res* 305: 33-41
- [16] Kang HS, Habib M, Chan J, et al. (2005) A paradoxical role for IFN- γ in the immune properties of mesenchymal stem cells during viral challenge. *Experimental hematology* 33: 796-803
- [17] Krampera M (2006) Role for interferon- γ in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* 24: 386-398
- [18] Meisel R, Zibert A, Laryea M, Gobel U, Daubener W, Dilloo D (2004) Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* 103: 4619-4621
- [19] Sato K, Ozaki K, Oh I, et al. (2007) Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. *Blood* 109: 228-234
- [20] Gieseke F, Schütt B, Viebahn S, et al. (2007) Human multipotent mesenchymal stromal cells inhibit proliferation of PBMCs independently of IFN γ R1 signaling and IDO expression. *Blood* 110: 2197-2200
- [21] Djouad F, Charbonnier L-M, Bouffi C, et al. (2007) Mesenchymal Stem Cells Inhibit the Differentiation of Dendritic Cells Through an Interleukin-6-Dependent Mechanism. *Stem Cells* 25: 2025-2032
- [22] Ren G, Su J, Zhang L, et al. (2009) Species Variation in the Mechanisms of Mesenchymal Stem Cell-Mediated Immunosuppression. *Stem Cells* 27: 1954-1962
- [23] Aggarwal S, Pittenger M (2005) Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 105: 1815-1822

- [24] Chabannes D, Hill M, Merieau E, et al. (2007) A role for heme oxygenase-1 in the immunosuppressive effect of adult rat and human mesenchymal stem cells. *Blood* 110: 3691-3694
- [25] Di Nicola M, Carlo-Stella C, Magni M, et al. (2002) Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 99: 3838-3843
- [26] Djouad F, Charbonnier L, Bouffi C, et al. (2007) Mesenchymal stem cells inhibit the differentiation of dendritic cells through an interleukin-6-dependent mechanism. *Stem Cells* 25: 2025-2032
- [27] Jiang X, Zhang Y, Liu B, et al. (2005) Human mesenchymal stem cells inhibit differentiation and function of monocytederived dendritic cells. *Blood* 105: 4120-4126
- [28] Nasef A, Mazurier C, Bouchet S, et al. (2008) Leukemia inhibitory factor: role in human mesenchymal stem cells mediated immunosuppression. *Cell Immunol* 253: 16-22
- [29] Nasef A, Zhang Y, Mazurier C, et al. (2009) Selected Stro-1-enriched bone marrow stromal cells display a major suppressive effect on lymphocyte proliferation. *Int J Lab Hematol* 31: 9-19
- [30] Nemeth K, Leelahavanichkul A, Yuen P, et al. (2009) Bone marrow stromal cells attenuate sepsis via prostaglandin E2-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 15: 42-49
- [31] Raffaghello L, Bianchi G, Bertolotto M, et al. (2008) Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche. *Stem Cells* 26: 151-162
- [32] Selmani Z, Naji A, Zidi I, et al. (2008) Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4⁺CD25^{high} Foxp3⁺ regulatory T cells. *Stem Cells* 26: 212-222
- [33] Spaggiari G, Abdelrazik H, Becchetti F, Moretta L (2009) MSCs inhibit monocytederived DC maturation and function by selectively interfering with the generation of immature DCs: central role of MSC-derived prostaglandin E2. *Blood* 113: 6576-6583
- [34] Xu G, Zhang Y, Zhang L, Ren G, Shi Y (2007) The role of IL-6 in inhibition of lymphocyte apoptosis by mesenchymal stem cells. *Biochem Biophys Res Commun* 361: 745-750
- [35] Lai RC, Arslan F, Lee MM, et al. (2010) Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* 4: 214-222
- [36] Bruno S, Grange C, Deregibus MC, et al. (2009) Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. *J Am Soc Nephrol* 20: 1053-1067
- [37] Ryan JM, Pettit AR, Guillot PV, Chan JK, Fisk NM (2013) Unravelling the pluripotency paradox in fetal and placental mesenchymal stem cells: Oct-4 expression and the case of The Emperor's New Clothes. *Stem Cell Rev* 9: 408-421
- [38] Thery C, Ostrowski M, Segura E (2009) Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol* 9: 581-593
- [39] Wubbolts R, Leckie RS, Veenhuizen PT, et al. (2003) Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *The Journal of biological chemistry* 278: 10963-10972
- [40] de Gassart A, Geminard C, Fevrier B, Raposo G, Vidal M (2003) Lipid raft-associated protein sorting in exosomes. *Blood* 102: 4336-4344
- [41] Zakharova L, Svetlova M, Fomina AF (2007) T cell exosomes induce cholesterol accumulation in human monocytes via phosphatidylserine receptor. *Journal of Cellular Physiology* 212: 174-181
- [42] Keller S, Konig AK, Marme F, et al. (2009) Systemic presence and tumor-growth promoting effect of ovarian carcinoma released exosomes. *Cancer Lett* 278: 73-81
- [43] Carmo A, Pedro M, Silva E, Knobel E, Laurindo F, Janiszewski M (2003) Platelet-derived exosomes: a new vascular redox signaling pathway. *Critical Care* 7: P117
- [44] Heijnen HF, Schiel AE, Fijnheer R, Geuze HJ, Sixma JJ (1999) Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood* 94: 3791-3799
- [45] Raposo G, Nijman HW, Stoorvogel W, et al. (1996) B lymphocytes secrete antigen-presenting vesicles. *The Journal of experimental medicine* 183: 1161-1172
- [46] Zitvogel L, Regnault A, Lozier A, et al. (1998) Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med* 4: 594-600
- [47] Raposo G, Tenza D, Mecheri S, Peronet R, Bonnerot C, Desaymard C (1997) Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon

- degranulation. *Molecular Biology of the Cell* 8: 2631-2645
- [48] Peters PJ, Geuze HJ, Van Der Donk HA, et al. (1989) Molecules relevant for T cell-target cell interaction are present in cytolytic granules of human T lymphocytes. *European Journal of Immunology* 19: 1469-1475
- [49] Fevrier B, Vilette D, Archer F, et al. (2004) Cells release prions in association with exosomes. *Proc Natl Acad Sci U S A* 101: 9683-9688
- [50] Wolfers J, Lozier A, Raposo G, et al. (2001) Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nature Medicine* 7: 297-303
- [51] Sokolova V, Ludwig A-K, Hornung S, et al. (2011) Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy. *Colloids and Surfaces B: Biointerfaces* 87: 146-150
- [52] Clayton A, Al-Taei S, Webber J, Mason MD, Tabi Z (2011) Cancer Exosomes Express CD39 and CD73, Which Suppress T Cells through Adenosine Production. *The Journal of Immunology* 187: 676-683
- [53] Sullivan R, Saez F, Girouard J, Frenette G (2005) Role of exosomes in sperm maturation during the transit along the male reproductive tract. *Blood Cells, Molecules, and Diseases* 35: 1-10
- [54] Admyre C, Grunewald J, Thyberg J, et al. (2003) Exosomes with major histocompatibility complex class II and co-stimulatory molecules are present in human BAL fluid. *European Respiratory Journal* 22: 578-583
- [55] Pisitkun T, Shen RF, Knepper MA (2004) Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A* 101: 13368-13373
- [56] Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C (2005) Exosomal-like vesicles are present in human blood plasma. *International Immunology* 17: 879-887
- [57] Simpson RJ, Jensen SS, Lim JW (2008) Proteomic profiling of exosomes: current perspectives. *Proteomics* 8: 4083-4099
- [58] Thery C, Zitvogel L, Amigorena S (2002) Exosomes: composition, biogenesis and function. *Nat Rev Immunol* 2: 569-579
- [59] Stoorvogel W, Kleijmeer MJ, Geuze HJ, Raposo G (2002) The biogenesis and functions of exosomes. *Traffic* 3: 321-330
- [60] Andre F, Scharz NE, Movassagh M, et al. (2002) Malignant effusions and immunogenic tumour-derived exosomes. *Lancet* 360: 295-305
- [61] Dai S, Wan T, Wang B, et al. (2005) More efficient induction of HLA-A*0201-restricted and carcinoembryonic antigen (CEA)-specific CTL response by immunization with exosomes prepared from heat-stressed CEA-positive tumor cells. *Clin Cancer Res* 11: 7554-7563
- [62] Clayton A, Mitchell JP, Court J, Mason MD, Tabi Z (2007) Human tumor-derived exosomes selectively impair lymphocyte responses to interleukin-2. *Cancer research* 67: 7458-7466
- [63] Taylor DD, Gercel-Taylor C (2008) MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecologic Oncology* 110: 13-21
- [64] Park JE, Tan HS, Datta A, et al. (2010) Hypoxic tumor cell modulates its microenvironment to enhance angiogenic and metastatic potential by secretion of proteins and exosomes. *Mol Cell Proteomics* 9: 1085-1099
- [65] Simons M, Raposo G (2009) Exosomes--vesicular carriers for intercellular communication. *Curr Opin Cell Biol* 21: 575-581
- [66] Raiborg C, Rusten TE, Stenmark H (2003) Protein sorting into multivesicular endosomes. *Curr Opin Cell Biol* 15: 446-455
- [67] Fang Y, Wu N, Gan X, Yan W, Morrell JC, Gould SJ (2007) Higher-order oligomerization targets plasma membrane proteins and HIV gag to exosomes. *PLoS Biol* 5: e158
- [68] Trajkovic K, Hsu C, Chiantia S, et al. (2008) Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* 319: 1244-1247
- [69] Pan BT, Johnstone RM (1983) Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell* 33: 967-978
- [70] Pegtel DM, Cosmopoulos K, Thorley-Lawson DA, et al. (2010) Functional delivery of viral miRNAs via exosomes. *Proc Natl Acad Sci U S A* 107: 6328-6333
- [71] Faure J, Lachenal G, Court M, et al. (2006) Exosomes are released by cultured cortical neurones. *Mol Cell Neurosci* 31: 642-648
- [72] Lachenal G, Pernet-Gallay K, Chivet M, et al. (2011) Release of exosomes from differentiated neurons and its regulation by synaptic glutamatergic activity. *Mol Cell Neurosci* 46: 409-418
- [73] Bakhti M, Winter C, Simons M (2011) Inhibition of myelin membrane sheath formation by oligodendrocyte-derived exosome-like vesicles. *The Journal of biological chemistry* 286: 787-796

- [74] Vrijzen KR, Sluijter JPG, Schuchardt MWL, et al. (2010) Cardiomyocyte progenitor cell-derived exosomes stimulate migration of endothelial cells. *Journal of Cellular and Molecular Medicine* 14: 1064-1070
- [75] Hao S, Ye Z, Li F, et al. (2006) Epigenetic transfer of metastatic activity by uptake of highly metastatic B16 melanoma cell-released exosomes. *Experimental Oncology* 28: 126-131
- [76] Peinado H, Aleckovic M, Lavotshkin S, et al. (2012) Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med* 18: 883-891
- [77] Meckes DG, Jr., Shair KH, Marquitz AR, Kung CP, Edwards RH, Raab-Traub N (2010) Human tumor virus utilizes exosomes for intercellular communication. *Proc Natl Acad Sci U S A* 107: 20370-20375
- [78] Bernardo ME, Pagliara D, Locatelli F (2012) Mesenchymal stromal cell therapy: a revolution in Regenerative Medicine? *Bone Marrow Transplant* 47: 164-171
- [79] Rajendran L, Honsho M, Zahn TR, et al. (2006) Alzheimer's disease beta-amyloid peptides are released in association with exosomes. *Proc Natl Acad Sci U S A* 103: 11172-11177
- [80] Fevrier B, Vilette D, Laude H, Raposo G (2005) Exosomes: a bubble ride for prions? *Traffic* 6: 10-17
- [81] Porto-Carreiro I, Fevrier B, Paquet S, Vilette D, Raposo G (2005) Prions and exosomes: from PrPc trafficking to PrPsc propagation. *Blood cells, molecules & diseases* 35: 143-148
- [82] Bhatnagar S, Schorey JS (2007) Exosomes released from infected macrophages contain Mycobacterium avium glycopeptidolipids and are proinflammatory. *The Journal of biological chemistry* 282: 25779-25789
- [83] Lenassi M, Cagney G, Liao M, et al. (2010) HIV Nef is Secreted in Exosomes and Triggers Apoptosis in Bystander CD4+T Cells. *Traffic* 11: 110-122
- [84] Vallhov H, Gutzeit C, Johansson SM, et al. (2011) Exosomes containing glycoprotein 350 released by EBV-transformed B cells selectively target B cells through CD21 and block EBV infection in vitro. *J Immunol* 186: 73-82
- [85] Diederick D, Theo L, Chris HB, Guido J (2010) Exosomes as Biomarker Treasure Chests for Prostate Cancer. *European urology* 59: 823-831
- [86] Amado LC, Saliaris AP, Schuleri KH, et al. (2005) Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. *Proc Natl Acad Sci U S A* 102: 11474-11479
- [87] Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD (2002) Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* 105: 93-98
- [88] Valina C, Pinkernell K, Song YH, et al. (2007) Intracoronary administration of autologous adipose tissue-derived stem cells improves left ventricular function, perfusion, and remodelling after acute myocardial infarction. *European Heart Journal* 28: 2667-2677
- [89] Shake JG, Gruber PJ, Baumgartner WA, et al. (2002) Mesenchymal stem cell implantation in a swine myocardial infarct model: Engraftment and functional effects. *Annals of Thoracic Surgery* 73: 1919-1926
- [90] Schuleri KH, Feigenbaum GS, Centola M, et al. (2009) Autologous mesenchymal stem cells produce reverse remodelling in chronic ischaemic cardiomyopathy. *European Heart Journal* 30: 2722-2732
- [91] Timmers L, Lim S-K, Arslan F, et al. (2008) Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. *Stem Cell Research* 1: 129-137
- [92] Timmers L, Lim SK, Hofer IE, et al. (2011) Human mesenchymal stem cell-conditioned medium improves cardiac function following myocardial infarction. *Stem Cell Res* 6: 206-214
- [93] Xin H, Li Y, Buller B, et al. (2012) Exosome-mediated transfer of miR-133b from multipotent mesenchymal stromal cells to neural cells contributes to neurite outgrowth. *Stem Cells* 30: 1556-1564
- [94] Li T, Yan Y, Wang B, et al. (2013) Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis. *Stem Cells Dev* 22: 845-854
- [95] Lee C, Mitsialis SA, Aslam M, et al. (2012) Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. *Circulation* 126: 2601-2611
- [96] Reis LA, Borges FT, Simoes MJ, Borges AA, Sinigaglia-Coimbra R, Schor N (2012) Bone marrow-derived mesenchymal stem cells repaired but did not prevent gentamicin-induced acute kidney injury through paracrine effects in rats. *PLoS ONE* 7: e44092
- [97] Tomasoni S, Longaretti L, Rota C, et al. (2013) Transfer of growth factor receptor mRNA via exosomes unravels the regenerative effect of mesenchymal stem cells. *Stem Cells Dev* 22: 772-780

- [98] Ludwig AK, Kordelas L, Rebmann V, et al. (2012) Exosomes-From Bench to Bedside. *Klin Padiatr* 224: A6
- [99] Cignarelli A, Perrini S, Ficarella R, Peschechera A, Nigro P, Giorgino F (2012) Human adipose tissue stem cells: relevance in the pathophysiology of obesity and metabolic diseases and therapeutic applications. *Expert Rev Mol Med* 14: e19
- [100] Bassand JP, Danchin N, Filippatos G, et al. (2005) Implementation of reperfusion therapy in acute myocardial infarction. A policy statement from the European Society of Cardiology. *Eur Heart J* 26: 2733-2741
- [101] Bleumink GS, Knetsch AM, Sturkenboom MCJM, et al. (2004) Quantifying the heart failure epidemic: prevalence, incidence rate, lifetime risk and prognosis of heart failure. *European Heart Journal* 25: 1614-1619
- [102] Jennings RB, Sommers HM, Smyth GA, Flack HA, Linn H (1960) Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. *Arch Pathol* 70: 68-78
- [103] Knight DR (2007) Editorial overview: cardioprotective drugs for myocardial ischemic injury--a therapeutic area at risk. *Curr Opin Investig Drugs* 8: 190-192
- [104] Lai RC, Arslan F, Tan SS, 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
- [105] Arslan F, Lai RC, Smeets MB, 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
- [106] Lai RC, Yeo RW, Tan KH, Lim SK (2013) Mesenchymal stem cell exosome ameliorates reperfusion injury through proteomic complementation. *Regen Med* 8: 197-209
- [107] Jennings RB, Reimer KA (1981) Lethal myocardial ischemic injury. *Am J Pathol* 102: 241-255
- [108] Insete J, Garcia-Dorado D, Ruiz-Meana M, et al. (2002) Effect of inhibition of Na⁺/Ca²⁺ exchanger at the time of myocardial reperfusion on hypercontracture and cell death. *Cardiovascular Research* 55: 739-748
- [109] Garnier A, Fortin D, Deloménie C, Momken I, Veksler V, Ventura-Clapier R (2003) Depressed mitochondrial transcription factors and oxidative capacity in rat failing cardiac and skeletal muscles. *Journal of Physiology* 551: 491-501
- [110] Jennings RB, Reimer KA (1991) The cell biology of acute myocardial ischemia. *Annual Review of Medicine* 42: 225-246
- [111] Gurusamy N, Goswami S, Malik G, Das DK (2008) Oxidative injury induces selective rather than global inhibition of proteasomal activity. *Journal of Molecular and Cellular Cardiology* 44: 419-428
- [112] Rosano GM, Fini M, Caminiti G, Barbaro G (2008) Cardiac metabolism in myocardial ischemia. *Curr Pharm Des* 14: 2551-2562
- [113] Frank A, Bonney M, Bonney S, Weitzel L, Koeppen M, Eckle T (2012) Myocardial Ischemia Reperfusion Injury. *Seminars in Cardiothoracic and Vascular Anesthesia* 16: 123-132
- [114] Becker LB (2004) New concepts in reactive oxygen species and cardiovascular reperfusion physiology. *Cardiovasc Res* 61: 461-470
- [115] Li C, Jackson RM (2002) Reactive species mechanisms of cellular hypoxia-reoxygenation injury. *Am J Physiol Cell Physiol* 282: C227-241
- [116] Eltzschig HK, Eckle T (2011) Ischemia and reperfusion--from mechanism to translation. *Nat Med* 17: 1391-1401
- [117] Li X, Arslan F, Ren Y, 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
- [118] Lai RC, Yeo RW, Tan SS, et al. (2012) Mesenchymal Stem Cell Exosomes: The Future MSC-based Therapy? In: Chase LG, Vemuri MC (eds) *Mesenchymal Stem Cell Therapy*. Humana Press, New York
- [119] Lai RC, Tan SS, Teh BJ, et al. (2012) Proteolytic Potential of the MSC Exosome Proteome: Implications for an Exosome-Mediated Delivery of Therapeutic Proteasome. *Int J Proteomics* 2012: 971907
- [120] Yeo Y, Park K (2004) Control of encapsulation efficiency and initial burst in polymeric microparticle systems. *Arch Pharm Res* 27: 1-12
- [121] Parolini I, Federici C, Raggi C, et al. (2009) Micro-environmental pH is a key factor for exosome traffic in tumor cells. *The Journal of biological chemistry* 284: 34211-34222
- [122] Schrader J (1985) Mechanisms of ischemic injury in the heart. *Basic Res Cardiol* 80 Suppl 2: 135-139

- [123] Lian Q, Lye E, Suan Yeo K, et al. (2007) Derivation of clinically compliant MSCs from CD105+, CD24-differentiated human ESCs. *Stem Cells* 25: 425-436
- [124] Mathews DJ, Graff GD, Saha K, Winickoff DE (2011) Access to stem cells and data: persons, property rights, and scientific progress. *Science* 331: 725-727
- [125] Smith A (2011) 'No' to ban on stem-cell patents. *Nature* 472: 418
- [126] Chen TS, Arslan F, Yin Y, et al. (2011) Enabling a robust scalable manufacturing process for therapeutic exosomes through oncogenic immortalization of human ESC-derived MSCs. *Journal of Translational Medicine* 9: 47
- [127] Chen TS, Lai RC, Lee MM, Choo AB, Lee CN, Lim SK (2010) Mesenchymal stem cell secretes micro-particles enriched in pre-microRNAs. *Nucleic Acids Res* 38: 215-224
- [128] Denzer K, Kleijmeer MJ, Heijnen HF, Stoorvogel W, Geuze HJ (2000) Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *Journal of cell science* 113 Pt 19: 3365-3374
- [129] Zitvogel L, Regnault A, Lozier A, et al. (1998) Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell derived exosomes. *Nat Med* 4: 594-600
- [130] Lamparski HG, Metha-Damani A, Yao J-Y, et al. (2002) Production and characterization of clinical grade exosomes derived from dendritic cells. *Journal of Immunological Methods* 270: 211-226
- [131] Simpson RJ, Mathivanan S (2012) Extracellular microvesicles: The need for internationally recognised nomenclature and stringent purification criteria. *Journal of Proteomics and Bioinformatics* 5: 2
- [132] Clayton A, Court J, Navabi H, et al. (2001) Analysis of antigen presenting cell derived exosomes, based on immuno-magnetic isolation and flow cytometry. *Journal of Immunological Methods* 247: 163-174
- [133] Mathivanan S, Lim JWE, Tauro BJ, Ji H, Moritz RL, Simpson RJ (2010) Proteomics Analysis of A33 Immunoaffinity-purified Exosomes Released from the Human Colon Tumor Cell Line LIM1215 Reveals a Tissue-specific Protein Signature. *Molecular & Cellular Proteomics* 9: 197-208