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Mesenchymal Stem Cell Derived Secretome and Extracellular Vesicles for Acute Lung Injury and Other Inflammatory Lung Diseases

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Abstract

Introduction—Acute respiratory distress syndrome is a major cause of respiratory failure in critically ill patients. Despite extensive research into its pathophysiology, mortality remains high. No effective pharmacotherapy exists. Based largely on numerous preclinical studies, administration of mesenchymal stem or stromal cell (MSC) as a therapeutic for acute lung injury holds great promise, and clinical trials are currently underway. However, concern for the use of stem cells, specifically the risk of iatrogenic tumor formation, remains unresolved. Accumulating evidence now suggest that novel cell-free therapies including MSC-derived conditioned medium and extracellular vesicles released from MSCs might constitute compelling alternatives.

Areas covered—The current review summarizes the preclinical studies testing MSC conditioned medium and/or MSC extracellular vesicles as treatment for acute lung injury and other inflammatory lung diseases.

Expert opinion—While certain logistical obstacles limit the clinical applications of MSC conditioned medium such as the volume required for treatment, the therapeutic application of MSC extracellular vesicles remains promising, primarily due to ability of extracellular vesicles to maintain the functional phenotype of the parent cell. However, utilization of MSC extracellular vesicles will require large-scale production and standardization concerning identification, characterization and quantification.

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Keywords

Acute Lung Injury; Acute Respiratory Distress Syndrome; Exosomes; Extracellular Vesicles; Mesenchymal Stem Cells; Microvesicles

1. INTRODUCTION

In critically ill patients, acute respiratory distress syndrome (ARDS) constitutes a major cause of acute respiratory failure whose mortality rate remains as high as 40%[1]. Current therapeutic strategies are primarily supportive measures including lung-protective ventilation, conservative fluid management as well as early neuromuscular blockade and prone positioning in the most severe cases[2–7]. Initially triggered by either pathogen- or danger-associated molecular patterns detected by resident antigen-presenting cells, the pathophysiology of ARDS arises from complex crosstalks between the immune system and the alveolocapillary barrier leading to an acute proinflammatory response accompanied with increased lung protein permeability and formation of pulmonary edema. Due to impaired alveolar fluid clearance, pulmonary edema eventually results in impaired gas exchange and hypoxemia[8]. However, previous clinical trials, which utilized pharmacological strategies targeting either the inflammatory or fibrotic pathways, have largely yielded negative results, suggesting that this therapeutic approach was too simplistic. Due to its ability to attenuate the major abnormalities underlying acute lung injury (ALI), mesenchymal stem or stromal cells (MSC) have become a promising approach for ARDS.

Although originally identified in the bone marrow, MSC can be isolated from a variety of tissues, such as umbilical cord blood, Wharton's jelly, placenta, and adipose tissue. According to the International Society for Cellular Therapy, their characterization is generally based on 3 criteria: 1) Plastic adherence when cultured in standard tissue culture conditions; 2) Expression of CD105, CD73, and CD90 with no expression of CD45, CD34, CD14, CD11b, CD79a, CD19, or HLA-DR surface markers; 3) And differentiation into osteoblasts, adipocytes and chondroblasts *in vitro*[9]. Over the past decade, preclinical research into MSC-based therapy have grown tremendously due to, initially, the potential of MSC to differentiate into resident injured cells, and, more recently, the ability of MSCs to secrete soluble factors, such as growth factors, anti-inflammatory cytokines, and antimicrobial peptides, which can stabilize the alveolocapillary barrier, enhance alveolar fluid clearance, and decrease infection[8, 10–12]. In a double-blind randomized single-center trial, Zheng et al. found that intravenous administration of human MSC in 12 patients with ARDS was safe[10]. In another multi-center, open-label, dose-escalation, phase 1 clinical trial, Wilson et al. showed that intravenous administration of human MSC was well tolerated in 9 patients with ARDS[11]. Based on these promising results, a phase 2 clinical trial is currently underway.

Despite these encouraging results, questions still remain concerning the optimal dose, route, source, timing, and duration of MSC treatment. Further investigations are also needed to standardize cell-based therapy with MSC for quality control, bacteriological testing, viability, phenotype, and oncogenic potential. Although considered "immunopriviledged"

allowing allogeneic transplantation, concerns for adverse immune dysfunction including increased susceptibility to sepsis, cancer and autoimmune diseases still exists. To overcome some of the concerns, early studies demonstrating that MSC-derived conditioned medium (CM) recapitulated many of therapeutic properties of the parent cells have paved the way for the development of cell-free strategies based on using components of MSC-derived CM, such as soluble factors, extracellular vesicles (EV), and potentially organelles (Figure 1).

In this review, we summarize the biological rationale and the preclinical data available for the potential therapeutic use of MSC-derived CM or EV for ALI and other inflammatory lung diseases. To achieve this goal, we reviewed relevant articles published between 2005 and 2015, the proceedings of major relevant conferences and major reviews, identified by searches in Medline, Current Contents, and PubMed, and references from relevant articles.

2. MESENCHYMAL STEM CELLS SECRETOME FOR ACUTE LUNG INJURY AND OTHER INFLAMMATORY LUNG DISEASES

The biological rationales for using MSC derived secretome is largely based on three preclinical findings: 1) The vast majority of studies have demonstrated that the mechanisms underlying the therapeutic effect of MSCs was due to secretion of soluble factors[12]. In various models of ALI, MSC secretion of keratinocyte growth factor (KGF)[13–16] and angiopoietin-1[17] have been shown to protect the alveolar epithelium and endothelium from injury in terms of protein permeability and loss of alveolar fluid clearance[12]. These promising results with KGF in ALI[18 - 21] have been recently reinforced with the KARE (keratinocyte growth factor in acute lung injury to reduce pulmonary dysfunction) clinical trial, whose final results are still pending [ISRCTN95690673]. Other MSC soluble factors such as interleukin-10 (IL-10), prostaglandin-E_2 (PGE_2), or transforming growth factor-β (TGF-β) have shown beneficial effects in suppressing inflammation; 2) Most preclinical studies have shown low engraftment rates (<5%), demonstrating that MSC replacement of injured cells was not significant[18–23]; 3) Finally, several studies have demonstrated that cell-free MSC-derived CM recapitulated the therapeutic effects of MSC[24].

In a model of ALI induced by intra-tracheal (IT) lipopolysaccharide (LPS)[25], MSC-derived CM (concentrated 25x) administered IT 4 hours following the injury decreased the alveolar influx of inflammatory cells and prevented pulmonary edema formation in part by promoting an alternate anti-inflammatory M2 macrophage phenotype *via* insulin-like growth factor I secretion. In LPS-induced ALI in an *ex vivo* perfused human lung[14], Lee et al. found that IT administration of MSC-derived CM 1 hour following injury decreased inflammation, prevented the influx of neutrophils and prevented pulmonary edema by restoring lung protein permeability and increasing alveolar fluid absorption in the injured alveolus. The authors found that blocking KGF secretion by using a neutralizing antibody abrogated the therapeutic properties of MSC-derived CM.

In bleomycin-induced ALI[26], investigators demonstrated that MSC-derived CM attenuated the influx of inflammatory cells within the alveolar space, while reversing histological evidence of lung fibrosis. Anti-inflammatory and anti-fibrotic effects were found to be driven by the restoration of lung-resident MSCs accompanied by an inhibition of T cell

proliferation. Several investigators utilized hyperoxia-induced injury in a model of bronchopulmonary dysplasia (BPD) in mice or rats pups to study the therapeutic effects of MSC CM (concentrated 20–25x)[27–33]. Hyperoxic conditions were applied immediately following birth from 10[27] to 14[28–32] days, and MSC CM was given *via* the intraperitoneal (IP)[30], intravenous (IV)[27, 29], or IT[28, 32, 33] route once[27–29, 32, 33] or daily[30]. Most of these studies demonstrated beneficial properties of MSC-derived CM in terms of reducing lung inflammation and histological injury, restoring lung compliance, and preventing pulmonary hypertension, which is one cardinal feature of BPD. Several pathways were identified as responsible for the beneficial effects of MSC-derived CM in BPD, such as inhibition of macrophage stimulating factor-1[27] and monocyte chemoattractant protein-1, increase in osteopontin expression[27], suppression of proinflammatory cytokines (interleukin-6, interleukin-1β)[32], increase in stanniocalcin-1 and expression of other antioxidants[30], and angiogenesis[32]. Pierro et al. administered MSC-derived CM either during oxygen exposure or 14 days following the hyperoxic exposure, enabling them to study respectively a preventive and treatment approach in rat pups[33]. Interestingly, in both models, MSC-derived CM was capable of decreasing lung inflammation and mean linear intercept, while increasing septal counts, lung compliance, and enhancing lung histology by attenuating the main features of BPD. Regarding pulmonary hypertension, the authors found that both pulmonary arterial remodeling and right ventricular hypertrophy, as assessed through the media wall thickness and the Fulton index, were prevented or fully reversed in the group of animals treated with MSC-derived CM.

Aside from ALI, MSC-derived CM have also showed promising results in asthma[34] and chronic emphysema[35, 36], in terms of reducing inflammation and histological damage within the bronchoalveolar airspace and lung parenchyma. In both acute and chronic ovalbumin-induced asthma model in mice, Ionescu et al. showed that MSC-derived CM attenuated inflammatory cells infiltrate into the alveolar space, restored the bronchodilator response to salbutamol, suppressed the increase in both dynamic lung resistance and elastance, and reduced airway smooth muscle layer thickening and peribronchial inflammatory infiltrate[34]. The beneficial effects of MSC-derived CM were partially explained by the restoration of a regulatory T cell subset overexpressing IL-10 and the induction of an emerging subset of IL-10 secreting monocytes-macrophages[34]. In a rat model of emphysema induced by cigarette smoke exposure, MSC-derived CM improved lung histology with a lower mean linear intercept, a higher lung vasculature density, and a lower right ventricular systolic pressure[35].

In summary, these findings strongly suggested that MSC-derived CM was capable of recapitulating the therapeutic effects of MSC in ALI and other inflammatory lung diseases through the activation of anti-inflammatory, pro-survival, and anti-apoptotic pathways. However, using MSC-derived CM as a therapeutic has limitations due to the lack of standardization in terms of the preconditioning process, which yields the MSC CM, as well as the optimal therapeutic dose, timing, and route of administration. For example, since the manner of preconditioning of MSCs may potentially impact the secretome, the best preconditioning protocol aside form serum starvation is unknown. Hypoxic preconditioning of the MSC has been shown to yield CM with higher levels of the antioxidant

stanniocalcin-1[30]. Even the optimal duration of serum deprivation is still debated in the literature, ranging from 12 to 72 hours. Similarly, the most potent concentration as a therapeutic of the MSC CM is unknown, making comparisons between studies difficult. Currently, the potential use of MSC CM in clinical trials is limited more so than the use of the stem cells due to a lack of standardization of the CM.

3. MESENCHYMAL STEM CELLS EXTRACELLULAR VESICLES FOR ACUTE LUNG INJURY AND OTHER INFLAMMATORY LUNG DISEASES

Although many soluble factors have been identified in the MSC CM with reparative properties, much is still unknown of the constituents of the CM. Recently, EVs have been identified in the MSC CM with therapeutic properties in multiple organ injury models, including ALI.

A. Definition and Characterization of Extracellular Vesicles

Once considered cellular debris, a growing body of evidence now demonstrates that secreted EV participates in human physiology and diseases. Many cells release EV into the extracellular environment in response to diverse physiological, pathophysiological, or external stimulus. Vesicles can be detected in cell culture supernatants and in diverse biological fluids such as blood, urine, sputum, synovial fluid, pleural effusion, breast milk, ascites and organ tissue, or cavity (e.g., alveolar space). Encapsulating a broad array of bioactive molecules such as proteins, peptides, mRNAs, microRNAs, and DNA, they can influence multiple biological signaling pathways of the recipient cells. In preclinical studies, EVs have been studied as potential biomarkers in multiple diseases and syndromes, and, more recently, as possible therapeutic vectors of bioactive molecules[16, 37].

1. Nomenclature Defined by Size, Morphology, and Biogenesis—Although there is no broad agreement on the classification of vesicles, in this review, we used the term EV in accordance with the recommendations of the International Society for Extracellular Vesicles, as an umbrella term encompassing exosomes, microvesicles (also referred to as ectosomes, shedding vesicles, microparticles, plasma membrane-derived vesicles, or exovesicles), and apoptotic bodies (also called apobodies), which are differentiated by their size and morphology as well as their biogenesis and secretion mechanisms[38, 39] (Figure 2).

Exosomes are 20 to 100 nm in size and are characterized by their endosomal origin, since they are specifically formed through the fusion of multivesicular endosomes with the cell membrane[40–44]. Their release is dependent on cytoskeleton activation, not on calcium influx. Exosomes contain enriched amounts of endosomal markers, including tetraspanins (CD63, CD81, CD9), heat-shock proteins (Hsp60, Hsp70 and Hsp90), ALG-2 interacting protein X (Alix), tumor susceptibility gene 101 (Tsg101), MHC classes I and II, and express low amounts of phosphatidylserine. In addition, annexins and clathrin are frequently present in exosomes. Most importantly, their molecular cargo includes proteins and RNAs that are specific to their cell source and their pathophysiological state[45, 46]. Two web-based

compendium of proteins and RNAs found in exosomes are freely accessible at Exocarta[47] (http://www.exocarta.org) or Vesiclepedia[48] (http://www.microvesicles.org).

Microvesicles are formed by direct budding off the plasma membrane and are larger than exosomes (100 to 1000 nm)[49–51] (Figure 2). They display a large amount of phosphatidylserine, cholesterol, proteins associated with lipid rafts, and they are enriched in cholesterol, sphingomyelin and ceramide. Microvesicles formation is dependent upon both cytoskeleton activation and calcium intracellular concentration. Both exosomes and microvesicles are constitutively released by multiple cell types whether upon physiological stimulus or in response to injury (Figure 2). They contain multiple cellular components that can drive cell-to-cell communication through the transfer of bioactive molecules including endosome-associated proteins, membrane proteins, lipids, and genetic material (e.g., mRNA, and microRNA)[50–53]. MSC-derived EV biogenesis has been shown to be regulated by cross-talk between MSC and their surrounding microenvironment. Thus, extracellular conditions, such as hypoxia or inflammation, influence molecular packaging into EVs, and impact their functional properties[54, 55]. Both exosomes and microvesicles interact with their target cells *via* either ligand-receptor signaling pathways or internalization by phagocytosis, endocytosis, and direct membrane fusion (Figure 2). These uptake mechanisms lead ultimately to the deliver of their molecular cargo to the recipient cells[53].

Apoptotic bodies are generally > 1000 nm in size that is released from cells undergoing programmed cell death. They contain potentially toxic or immunogenic cellular components, such as DNA fragments, non-coding RNAs, and cell organelles, which are destined to be cleared through phagocytosis.

Even though a growing body of research suggests that both endogenous lung progenitor cells[56, 57] and lung resident MSCs[58–60] coexist within specific lung niches, EV fraction released from these endogenous stromal cells still remains to be investigated. While both fate and phenotype of these resident lungs cells have been shown to be essentially driven by the microenvironment, studies on *in vivo* release of EV from endogenous MSC are still lacking, and whether EV production differs *in vivo* from *in vitro* conditions still remains unknown. We therefore focused our review on the potential therapeutic use of EV specifically released from exogenous MSC.

2. Isolation Methods of Extracellular Vesicles—Current methods used to isolate EV include ultracentrifugation, filtration, immune-affinity isolation, and polymeric precipitation[38]. Although the most commonly used technique and often considered as the gold-standard, the use of ultracentrifugation has certain pitfalls and limits, such as the amount of contaminants and length of the isolation procedure. In the literature, differences in EV yield, purity, length of the procedure, and product heterogeneity have been reported with ultracentrifugation alone, and some combination of techniques has been proposed to improve their performance.

3. Quantification of Extracellular Vesicles—Developing accessible methods for identifying and quantifying EV have improved substantially. However, agreeing on a standard unit of quantity for EV has remained challenging. Five methods are commonly

used: optical single particle tracking, flow cytometry, electron microscopy, protein concentration, and cell count. Electron microscopy remains a gold-standard in assessing morphology, size and for mapping of organelle specific markers (immuno-EM). However, this technique will not provide the total particle count or concentration. Using cell count to quantify the EV constitutes a simple option, which enables researchers to compare the level of biological activity of a certain quantity of cell-derived EV with its cell-equivalent in terms of therapeutic potency.

Since they provide complementary information on quantity, types and size of particles, or protein content, using a combined approach based on several methods remains the optimal way of quantifying EV yield. Research in exosomes or microvesicles derived from MSCs have used all of these techniques.

B. Therapeutic Properties of Mesenchymal Stem Cells Extracellular Vesicles

In various organ injury models, MSC derived vesicles, whether exosomes or microvesicles, have been shown to be as potent as the parent stem cell as a therapeutic (Figure 3). The mechanisms have been primarily mediated through the transfer of the content from the vesicles to the recipient cells, changing the function and/or phenotype of the recipient cell. Multiple recent studies have presented preclinical data addressing the reparative and regenerative properties of MSC vesicles following injuries to the kidney, heart, liver, brain as well as following hind limb ischemia injury[61]. Since these experimental injury models share common signaling pathways with ALI, leading to either extra-pulmonary or pulmonary acute organ dysfunction, understanding the therapeutic effects of MSC derived vesicles in such models may yield insights into their potential effect in ALI.

1. MSC Vesicles for Kidney Injury—MSC derived vesicles have been studied as a therapeutic in acute kidney injury (AKI) models [62 – 73] induced by cisplatin[62, 63], glycerol[69], ischemia-reperfusion (I/R)[70], nephrectomy[64], and drug toxicity (gentamicin)[65]. Compared to injury, MSC exosomes or microvesicles improved renal function and reduced the extent of kidney damage. In an AKI model in severe combined immunodeficiency mice, Bruno et al.[69] found that MSC microvesicle (MV) administration improved recovery from AKI in part by preventing apoptosis and increasing renal tubular epithelial cell proliferation. A single administration of MSC MV immediately following cisplatin[62] or I/R[70] induced AKI alleviated inflammation, mitigated renal cell apoptosis, and enhanced proliferation of the renal epithelial cells. The MV also accumulated at the site of renal injury[71]. Multiple injections of MSC MVs further improved renal function and morphology, abrogated renal fibrosis, and decreased mortality.

Overall, MVs were found to mimic the beneficial effects of MSCs, modulating T-cells as well as innate immune cell functions[54]. The effects appeared to be mediated in part by the transfer of RNA by MSC MVs to the injured renal epithelium, as indicated by the loss of reparative effects after RNase pretreatment of the MVs[40, 69, 72]. Specifically, Bruno et al. demonstrated that MSC MVs shuttled RNA associated with transcription, proliferation and immune regulation to the injured epithelium. The effective transfer of MV derived microRNA and mRNA and the translation of MV-shuttled mRNA into proteins within

recipient cells were shown both *in vitro* and *in vivo*[52, 69]. Tomasoni et al. reported that horizontal transfer of insulin-like growth factor-1 receptor (IGF-1R) mRNA to tubular cells also contributed to the powerful renoprotection of MSCs observed *in vivo*[73]. MSC MVs conferred an anti-apoptotic phenotype necessary for tissue repair by inducing the expression of anti-apoptotic genes (Bcl-XL, Bcl2 and BIRC8) in renal tubular epithelial cells while simultaneously down-regulating pro-apoptotic genes (Casp1, Casp8 and LTA)[62]. In addition, MSC MVs stimulated renal cell proliferation by inducing the phosphorylation and subsequent activation of extracellular regulated kinase (ERK)½ [63]. The authors found that MSC MVs expressed several adhesion molecules such as CD44, CD29 (β1-integrin), α4 and α5 integrins and CD73, , which were critical for the incorporation of the MVs into target cells[69, 72, 74, 75]. These data demonstrated the ability of MSC MVs to modulate simultaneously several different pathways to stimulate renal repair and/or regeneration.

2. MSC Vesicles for Cardiac Injury—The therapeutic effects of MSC vesicles have also been reported in several experimental models of myocardial I/R injury[76–80]. Lai et al. found that conditioned medium, primarily the exosome fraction, from human embryonic stem cell-derived MSCs significantly reduced infarct size in pig and mouse models of I/R injury[77, 81]. Administration of purified MSC exosomes before reperfusion significantly reduced infarct size and improved left ventricular function.

Based on the preclinical data, several mechanisms for the therapeutic effect of MSC exosomes in myocardial I/R injury have been postulated: (1) Exosomes contained membrane proteins that have significant binding affinity to other ligands on cell membranes or to the extracellular matrix. Specifically, integrins could home exosomes to cardiomyocytes that expressed ICAM1, a ligand for integrins, after myocardial I/R injury[82] or to VCAM-1 on endothelial cells[83]. Tetraspanin proteins, which function primarily to mediate cellular penetration, invasion and fusion events[84], could also facilitate cellular uptake of exosomes. Interestingly, it was observed that the efficiency of exosome uptake correlated directly with intracellular and micro-environmental acidity[85]. This may be a mechanism by which MSC exosomes exert their cardio-protective effects on ischemic cardiomyocytes that have a low intracellular pH[86]; (2) Many of the proteins in the exosomes are enzymes. Since enzyme activities are catalytic rather than stoichiometric and are dictated by their microenvironment (e.g., substrate concentration or pH), the enzyme-based therapeutic activities of exosomes could be activated or attenuated according to the release of injury-associated substrates[81]. For example, Lai et al. found by mass spectrometry and antibody array a significant clustering of enzymes involved in glycolysis in MSC exosomes. These enzymes from MSC exosomes if given as therapy could ameliorate the glycolytic deficit and potentially increase glycolytic flux and ATP production in the reperfused myocardium[87]. The intracellular transfer of glycolytic enzymes from MSC exosomes across the plasma membrane is likely to be sufficient for the enzyme to exert their biochemical activity as glycolysis is a cytosolic process unlike fatty acid (FA) oxidation and the tricarboxylic acid (TCA) cycle, which are mitochondrial processes. An important corollary of this hypothesis is that MSC exosomes increased ATP production in reperfused myocardium and/or in cells where oxidative phosphorylation was inhibited[76, 87]; (3) Activation of pro-survival protein kinases, such as Akt and ERK½, which prevent apoptosis, are protective against myocardial I/R injury

when stimulated at the time of myocardial reperfusion[88, 89]. These kinases are termed the reperfusion injury salvage kinases (RISK)[90]. Lai et al. postulated that CD73, which was present on the surface of MSC exosomes, was the most likely protein candidate to overcome the pro-apoptotic milieu of the reperfused myocardium through the activation of RISKs[87]; (4) And MSC exosomes may reduce the levels of misfolded or oligomerized proteins in reperfused hearts through the transfer of functionally active 20S proteasomes, which is responsible for the degradation of ~90% of all intracellular protein damaged by oxidation[91]. In addition, CD59 (protectin), a widely expressed glycosylphosphatidylinositol (GPI)-anchored membrane protein, is also present on MSC exosomes[92]; this molecule inhibits the terminal pathway of complement preventing the formation of the membrane attack complex (MAC) and complement-mediated lysis. Moreover, recent studies found that microRNAs associated with exosomes also played an important role in cardio-protection, such as the anti-apoptotic effect of miR-22[93] which directly targeted methyl CpG binding protein 2 (Mecp2) and reduced the expression of p53, a modulator of apoptosis, via miR-221[94].

3. MSC Vesicles for Liver Injury—Two studies have focused on the therapeutic effects of MSC exosomes in a mouse model of liver injury. Using a tetrachloride (CCL_4)-induced liver injury model[95, 96], the investigators demonstrated that MSC exosomes ameliorated liver fibrosis by inhibiting the epithelial-to-mesenchymal transition of hepatocytes and collagen production. Exosomes were found to significantly restore serum aspartate aminotransferase activity and decrease collagen type I/III and TGF-β1 and the phosphorylation of Smad2, inactivating the TGF-β1/Smad signaling pathway. In another study[96], Tan et al. found that MSC exosomes elicited protective effects in acetaminophen and hydrogen peroxide-induced liver injury primarily through an increase in hepatocyte proliferation, as demonstrated with proliferating cell nuclear antigen (PCNA) elevation and higher cell viability. The increased survival rate was associated with upregulation of priming-phase genes involved during liver regeneration, which subsequently led to higher expression of proliferation proteins (PCNA and cyclin D1) and the anti-apoptosis gene, Bcl-xL, and higher expression of signal transducer and activator of transcription 3 (STAT3). Surprisingly, however, the therapeutic effects of MSC exosomes was not through modulation of oxidative stress during hepatic injury.

4. MSC Vesicles for Neural Injury—The therapeutic role of MSC exosomes has been studied in a few models of neural ischemia [97 – 98]. Xin et al. demonstrated that functional microRNAs, such as miR-133b which is involved in regeneration of motor neuron axons[98], are transferred from MSC to injured neural cells via exosomes, and that transfer of microRNAs promoted neurite remodeling and functional recovery following stroke in rats[98, 99]. In a follow-up study[100], the investigators administered cell-free MSC exosomes to rats subjected to middle cerebral artery occlusion and investigated improvement of functional recovery and enhancement of neurite remodeling, neurogenesis, and angiogenesis. By evaluating functional recovery with a Foot-fault test[101] and a modified neurologic severity score[102], MSC exosomes treatment was associated with significant functional improvements following middle cerebral artery occlusion. MSC exosome treatment also significantly increased axonal density and synaptophysin-positive areas along

the ischemic boundary zone of the cortex and striatum as well as the number of newly formed doublecortin (a marker of neuroblasts) and von Willebrand factor (a marker of endothelial cells) cells.

In summary, multiple extra-pulmonary organ injuries share common pathophysiological pathways with inflammatory lung diseases including ALI. Therefore, understanding the therapeutic effects of MSC derived vesicles in these extra-pulmonary injury models may yield insights into its effects in ALI, especially in terms of suppressing inflammation, preventing apoptosis or enhancing cellular energetics as well as in ALI specific pathologies such as decreasing endothelial permeability or enhancing alveolar epithelial fluid absorption.

C. Therapeutic Properties of Mesenchymal Stem Cell Extracellular Vesicles in Acute Lung Injury and Other Inflammatory Lung Diseases

To date, only a few groups have studied the therapeutic effects of MSC vesicles in acute inflammatory lung diseases such as ALI[16, 103–105], pulmonary artery hypertension (PAH)[106, 107] and asthma[108]. Although the mechanisms of action have not been fully defined, these groups have demonstrated that MSC vesicles are as potent as their parent stem cells as therapy (Figure 4).

Zhu et al. demonstrated a biologic effect of MVs derived from human bone marrow MSCs in a mouse model of endotoxin-induced ALI[16]. Treatment with MSC MVs was effective in restoring lung protein permeability, reducing inflammation (e.g., the influx of neutrophils and elevation of macrophage inflammatory protein-2 levels), and preventing the formation of pulmonary edema in the injured alveolus. KGF mRNA knockdown partially abrogated the therapeutic effects of MSC MVs, suggesting that KGF protein expression was important for the underlying mechanism. Previously, KGF secretion by MSC was found to be involved in restoring both vectorial ion and fluid transport in injured human alveolar epithelial type II cells and in increasing the antimicrobial properties of monocytes/macrophages[15].

Monsel et al.[103], using a model of *Escherichia coli* pneumonia in mice, demonstrated that administration of MSC MVs improved survival and mitigated lung inflammation, protein permeability, and bacterial growth. The results suggested several potential mechanisms underlying the beneficial effects of MSC MVs: 1) Enhancement of monocyte phagocytosis of bacteria, which could be further increased by pre-stimulation of MSC with a toll-like receptor 3 agonist prior to the release of MVs; 2) The transfer of mRNA for cyclooxygenase 2 (COX2), the key enzyme in prostaglandin E_2 (PGE_2) synthesis, from MSC MVs to activated monocytes with a resultant increase in PGE_2 secretion, causing a shift in monocytes toward an anti-inflammatory M2 phenotype. Surprisingly, the authors found no effect on CD163 or CD206 mRNA expression (M2 markers) in monocytes exposed to MSC MVs, suggesting that the M2 shift with MSC MVs may be partial; 3) MSC MVs restored ATP levels in injured ATII, suggesting a metabolic benefit; 4) In primary cultures of human monocytes or human alveolar type 2 cells, the uptake of MSC MVs using the CD44 receptor on MVs was essential for their therapeutic effects. The findings suggested that MSC MVs were as effective as the parent stem cells in ALI from severe bacterial pneumonia.

In an *ex vivo* human lung perfusion model of I/R seen in lung transplantation[15], Gennai et al.[105] found that MSC MVs increased alveolar fluid clearance (e.g., ability to absorb pulmonary edema fluid) in a dose-dependent manner, decreased lung weight gain following perfusion and ventilation, and improved airway and hemodynamic parameters compared to perfusion alone. Co-administration of MVs with anti-CD44 antibody attenuated these effects, suggesting a key role of the CD44 receptor in the internalization of the MVs into the injured host cell and its effect. However, MSC MVs did not decrease alveolar inflammation as assessed by BAL fluid TNF-α levels. Likewise, in terms of potential mechanisms, the authors found only a non-significant increase in angiopoietin-1 (Ang1) levels associated with a decrease in syndecan-1 levels, a component of the endothelial glycocalyx, suggesting that restoration of lung protein permeability may be critical[109].

In a model of hypoxia-induced PAH in mice, Lee et al.[106] demonstrated that exosomes mediated the cytoprotective effect of bone marrow derived MSC. Administration of MSC-derived exosomes protected against the elevation of right ventricular systolic pressure and the development of right ventricular hypertrophy (RVH) after three weeks of hypoxic exposure. Specifically, treatment with MSC exosomes interfered with the early hypoxic signal in the lung, suppressing inflammation, the up-regulation of hypoxia-induced mitogenic factor (HIMF), and alveolar macrophage activation. Whereas, surprisingly, exosomes-depleted conditioned media had no therapeutic effect, suggesting a limited role of soluble factors released by MSCs. In addition, MSC exosomes suppressed the hypoxic induction of STAT3 in primary cultures of pulmonary artery endothelial cells and upregulated miR-204 levels, interfering with the STAT3-miR-204-STAT3 feed-forward loop, and shifting the balance to an anti-proliferative state. Similarly, Chen et al.[107] demonstrated that MSC MVs decreased pulmonary pressure and right ventricular pressure and reduce RVH and pulmonary arteriole remodeling during the development of PAH in rats.

Moreover, MSC exosomes were found to have reparative effects in a preclinical model of allergic airway inflammation provoked by mucosal sensitization and challenge with aspergillus hyphal extract (AHE)[108]. In AHE-induced ALI model, the authors found that the reduction in soluble Th2- (IL-4 and IL-5) and Th17- (IL-17) associated cytokines in bronchoalveolar lavage fluid and in mixed lymphocyte cultures was accompanied by an increase in IFN-γ expression. This suggested that both syngeneic and xenogeneic administration of MSC EV was as effective as the cells themselves in mitigating Th2/Th17-mediated airways hyper-responsiveness (AHR), by shifting the Th2/Th17 inflammatory response towards a counter-regulatory Th1 response, and reducing lung inflammation. Interestingly, blocking the release of soluble mediators and of EVs with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDCI) completely abrogated the effects of human MSCs as compared to mouse MSCs, suggesting potential different mechanisms between mouse and human MSCs.

Lastly, using MSC as a therapeutic to prevent silica-induced lung inflammation and fibrosis, Phinney et al.[104] found that MSCs shed exosomes that modulated toll-like receptor signaling and cytokine secretion in macrophages, in part, by transfer of regulatory microRNAs; miR-451, known to suppress TNF and macrophage migration inhibitory factor,

was highly abundant in MSC-derived exosomes, suggesting that the possible transfer of miR-451 to and increased expression in macrophages inhibited TNF secretion in response to silica. The authors also demonstrated that MSC-derived exosomes prevented the recruitment of $Ly6C^{hi}$ monocytes and reduced secretion of pro-fibrotic IL-10 and TGFβ by these cells. Lastly, the author found that MSCs managed intracellular oxidative stress by the transfer of depolarized mitochondria by MSCs. The vesicles were engulfed and re-utilized by macrophages, resulting in enhanced bioenergetics. To achieve these transfers, MSCs loaded mitochondria in the cytoplasm into microtubule-associated protein 1 light chain 3 (LC3) containing MVs. These MVs expressed the endosomal sorting complex required for transport (ESCRT) associated proteins tumor suppressor gene 101 (TSG101) and arrestin domain-containing protein 1 (ARRDC1) and were extruded from cells in arrestin domain-containing protein 1-mediated MVs (ARMMs), which budded outwards directly from the plasma membrane where they were identified by macrophages.

Although the few preclinical studies are very promising, more research is needed to better understand the mechanisms underlying the therapeutic effects of MSC vesicles.

4. CONCLUSIONS

By maintaining the therapeutic advantages of MSCs without the risk of iatrogenic tumor formation or of pulmonary embolisms with intravenous administration, MSC-derived EV represent an attractive area of research for treating inflammatory lung diseases. Currently, there is one ongoing Phase 1 studying the therapeutic effect of MSC EVs in type 1 diabetes (NCT02138331). However, questions still remain concerning characterization, potency and the quantification techniques used with MSC EVs, making a direct comparison to MSCs difficult. In order for the field to advance significantly in terms of translation into clinical trials, the nomenclature used and the isolation and characterization of MSC EVs must be standardized, allowing comparisons between preclinical studies and against the gold standard, the stem cell.

5. EXPERT OPINION

The use of MSC-derived extracellular vesicles as a cell-free therapeutic in lung diseases offer several advantages compared to MSCs: 1) EVs are non-self-replicating, reducing the risk of iatrogenic tumor formation. In a typical clinical trial using MSCs, a patient may receive up to 400 million cells per dose, and screening for tumor formation will be followed by only a computed tomography of the chest, abdomen and pelvis; 2) EVs can be stored without DMSO at – 80ºC and remain biologically active. For most clinical trials, MSCs will be stored in liquid nitrogen with DMSO in a bone marrow transplant facility, limiting the number of patients who may benefit from stem cell-based therapy; 3) And lastly, MSC EVs do not express MHC I or II antigens, nor can it be induced to, allowing allogeneic transplantation. Although once considered immunopriviledged, MSCs can be induced to express higher levels of MHC II with inflammation [110], potentially leading to an immune reaction in the recipient. However, questions remain which must be addressed before translation into clinical use.

1. Large-Scale MSC Extracellular Vesicle Generation

Based on preclinical studies, the amount of MSC EVs needed to generate an equivalent effect as MSCs in lung injury is roughly 5–10x higher[16, 103, 104]. If the average therapeutic dose of MSC used ranges from 1 to 10 × 10^6 cells/kg per body weight, the number of MSCs required to generate enough EVs may be > 10 to 100 × 10^6 of MSC/kg, perhaps making the production costs prohibitive. Although MSC are relatively easy to expand using conventional tissue flasks and bioreactors, their growth in culture is finite and their biological properties may become altered with repeated passages. New batches of MSC will have to be periodically derived with significant impact on the costs of derivation, testing, and validation. Strategies such as MSC immortalization, therefore, by genetic modification and clonal isolation could be used to overcome this limitation although this would also raise safety issues[111]. Recently, Chen et al.[112] proposed a robust scalable manufacturing process for therapeutic EV through oncogenic immortalization of human embryonic stem cell (ESC)-derived MSC by transfection with the MYC gene. Another approach to scale up EV production could be the use of bioreactors to culture MSC[113]. Several studies have documented significant increases in EV yield from cells cultured in bioreactor systems when compared with conventional tissue culture flasks[114]. We should be mindful, however, that the different bioreactor culture conditions such as adequacy of oxygen supply, hydrodynamic shear stress, metabolic byproducts build-up, and pH balance would result in alterations to EV content that may impact on therapeutic efficacy[115–118].

In the future, although several high throughput techniques such as centrifugation combined with either ultrafiltration or high pressure liquid chromatography or differential ultracentrifugation combined with sugar cushion have been proposed as viable manufacturing processes, more techniques for large-scale EV production need to be developed.

2. Issues of Potency

Techniques available used in preclinical studies for characterizing MSC EV such as proteomics, mass spectrometry, or microarrays have evolved[37]. Unfortunately, there is still no gold standard to characterize MSC EVs used by investigators. More importantly, the methods used to precondition MSC to stimulate vesicle release such as serum starvation, hypoxia, inflammation, etc. will change the surface and intracellular content of the released vesicles. EV usually mirrors the phenotype of their parent cells, which can be skewed toward either a more pro-inflammatory or immunomodulatory state through different preconditioning protocols[119–122]. For example, in the study by Monsel et al.[103], EV-derived from MSC pretreated with TLR3 agonists exhibited both higher bactericidal property and the capacity to skew human monocytes towards a M2 anti-inflammatory state. How pre-treatment modulates the phenotype of the released vesicles will need to be determined. Soluble factors released by MSC have therapeutic efficacy in a variety of inflammatory disorders, and released vesicles do contain these proteins[15, 123–130]. However, the role of these proteins within the vesicles is unclear. In addition, the use of ultracentrifugation to isolate MSC EVs do not differentiate based on the size of the vesicles. Although both microvesicles and exosomes have therapeutic properties[62, 106], further studies are needed to determine the contribution of each in the overall therapeutic effect.

3. Risks of MSC EV Administration

Although MSC EVs clearly lack the potential to directly form tumors, this does not imply that MSC-EV administration to human subjects is without any risk of promoting neoplasia[131]. Roccaro et al.[132] isolated EV from BM-MSC derived from multiple myeloma (MM) patients, which was found to promote tumor growth and induce cell dissemination and metastasis to distant MM niches. The authors observed lower miR-15a expression in MM versus normal BM MSC derived EV; miR-15a is associated with tumor-suppressive properties, as shown by inhibition of cell proliferation in miR-15a overexpressing MM cells, both *in vitro* and *in* vivo[133]. In another study[134], MSC-EV co-implanted with SGC-7901 (human gastric cancer) cells increased tumor growth and angiogenesis when compared with SGC-7901 cells alone. However, Lee et al.[135] reported contradictory results suggesting that MSC-EV suppressed angiogenesis *in vitro* in tumor cells in a concentration-dependent manner and speculated that this inconsistency may be due to different tumor types or MSC heterogeneity. Clearly, how the microenvironment effects the phenotype of endogenous or exogenous MSCs, especially when comparing the differences in the microenvironment in cancer vs. inflammation, will need to be further elucidated.

ABBREVIATIONS

AHE	Aspergillus hyphal extract
AHR	Airways hyper-responsiveness
AKI	Acute kidney injury
Alix	ALG-2 interacting protein X
ALI	Acute lung injury
ARDS	Acute respiratory distress syndrome
BPD	Bronchopulmonary dysplasia
Casp	Caspase
CCL4	Tetrachloride
CD	Clusters of differentiation
CM	Conditioned medium
DNA	Deoxyribonucleic acid
ERK	Extracellular regulated kinase
ESC	Embryonic stem cell
EV	Extracellular vesicles
FA	Fatty acid

HIMF	Hypoxia-induced mitogenic factor
Hsp	Heat shock proteins
IGF-1R	Insulin-like growth factor-1 receptor
IL	Interleukin
IP	Intraperitoneal
I/R	Ischemia-reperfusion
IT	Intra-tracheal
IV	Intravenous
KGF	Keratinocyte growth factor
GPI	Glycosylphosphatidylinositol
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
microRNA	Micro ribonucleic acid
MM	Multiple myeloma
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
MV	Microvesicles
PAH	Pulmonary artery hypertension
PCNA	Proliferating cell nuclear antigen
PGE_2	Prostaglandin E2
RISK	Reperfusion injury salvage kinases
RVH	Right ventricular hypertrophy
STAT3	Signal transducer and activator of transcription 3
TCA	Tricarboxylic acid
TGF-β	Transforming growth factor-β
TSG	Tumor susceptibility gene

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(•) or of considrable interest

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HIGHLIGHTS

- MSC-derived conditioned medium (CM) recapitulated the therapeutic effects of MSC in acute lung injury (ALI) and other inflammatory lung diseases through activation of anti-inflammatory, pro-survival and anti-apoptotic pathways.
- MSC-derived CM as a therapeutic has limitations due to the lack of standardization in terms of the preconditioning process as well as the optimal therapeutic dose, timing and route of administration.
- By maintaining the therapeutic advantages of MSC without the inherent risk of iatrogenic tumor formation, MSC-derived extracellular vesicles (EV) represent an attractive area of research for treating inflammatory lung diseases, including ALI.
- The mechanisms underlying the therapeutic effect of MSC-derived EVs appears to derive from the transfer of it's content which include mRNA, microRNA, proteins, receptors, and possibly organelles to the injured tissue.
- Utilization of MSC-derived EV will require large-scale production and standardization concerning identification, characterization and quantification.

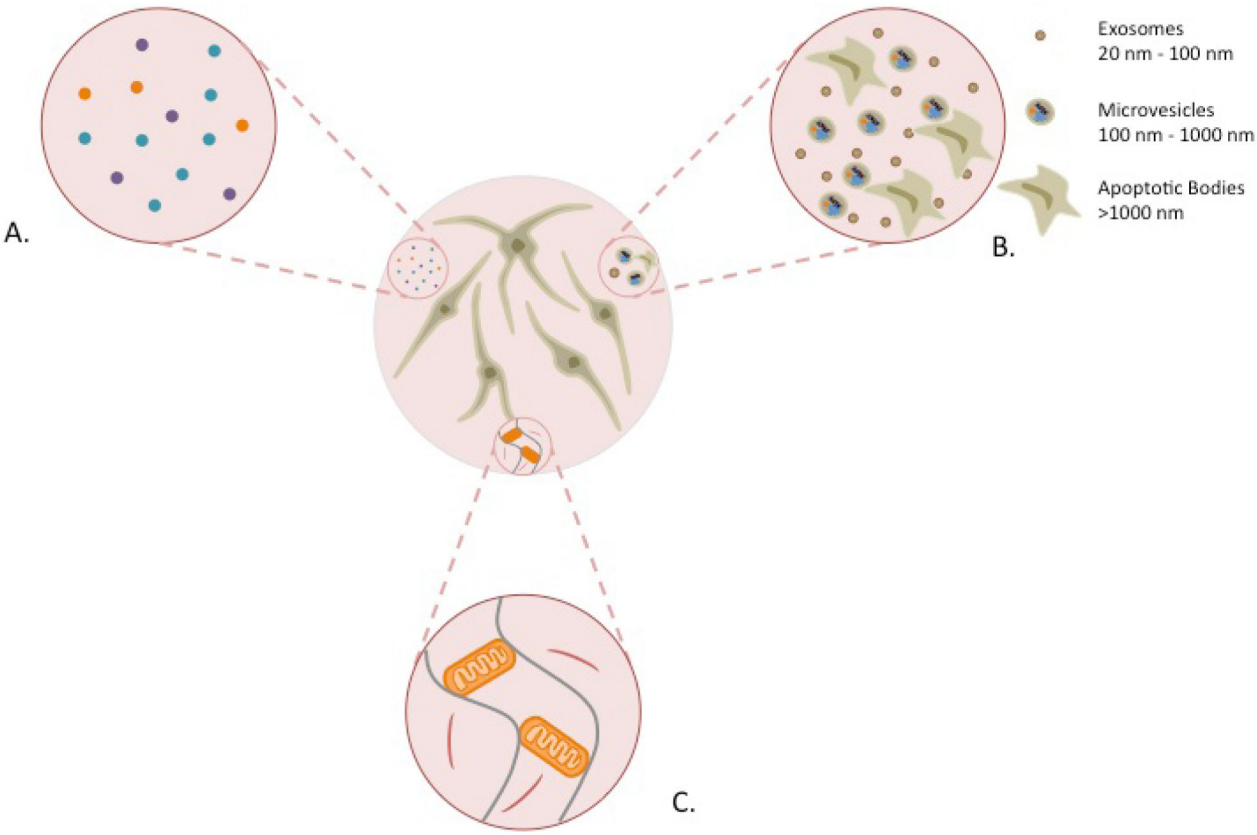

Figure 1. Components of Mesenchymal Stem Cell Derived Conditioned Medium with Reparative Properties

A) A wide array of immunomodulatory soluble factors with reparative properties is secreted by human MSCs, such as keratinocyte growth factor, angiopoietin-1, interleukin-10, prostaglandin-E_2 and transforming growth factor-β; **B**) No longer considered cellular debris, extracellular vesicles released by MSCs, which contain proteins, peptides, lipids, mRNAs, microRNAs and DNA, are biologically active and may participate in the therapeutic effect. Largely classified based on size, source and content, extracellular vesicles are comprised of exosomes, microvesicles and apoptotic bodies; **C**) MSCs are now recognized to be capable of transporting cellular organelles (e.g., mitochondria) to recipient cells through microtubules.

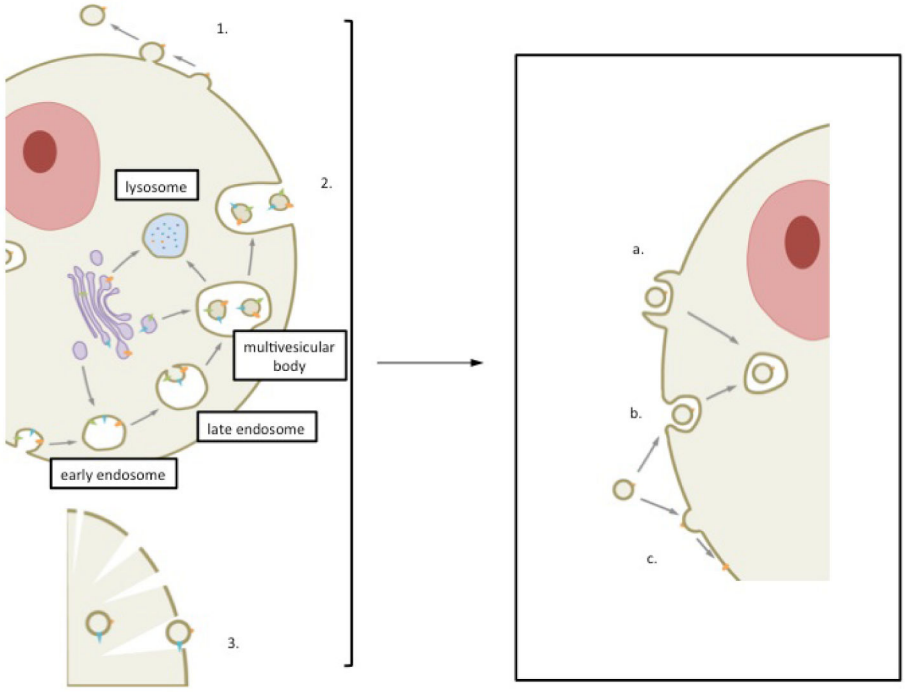

Figure 2. Extracellular Vesicles Biogenesis and Interactions with Recipient Cells

Biogenesis Extracellular vesicles originate from distinct intracellular compartments: **1**) Microvesicles which contain cytoplasmic molecules, are formed by direct budding off the plasma membrane into the extracellular space; **2**) Invagination of late endosomes, which is loaded with Golgi or cell surface-related molecules, forms multivesicular bodies that fuse with plasma membrane giving rise to exosomes; **3**) Apoptotic bodies are released from cells undergoing programmed cell death. They contain potentially toxic or immunogenic cellular components, such as DNA fragments, non-coding RNAs, and cellular organelles, which are destined to be cleared through phagocytosis. ***Interaction Between Extracellular Vesicles and Recipient Cells***. Internalization of extracellular vesicles leading to the release of their content within recipient cells can be mediated through (**a**) phagocytosis, (**b**) endocytosis, or (**c**) direct membrane fusion.

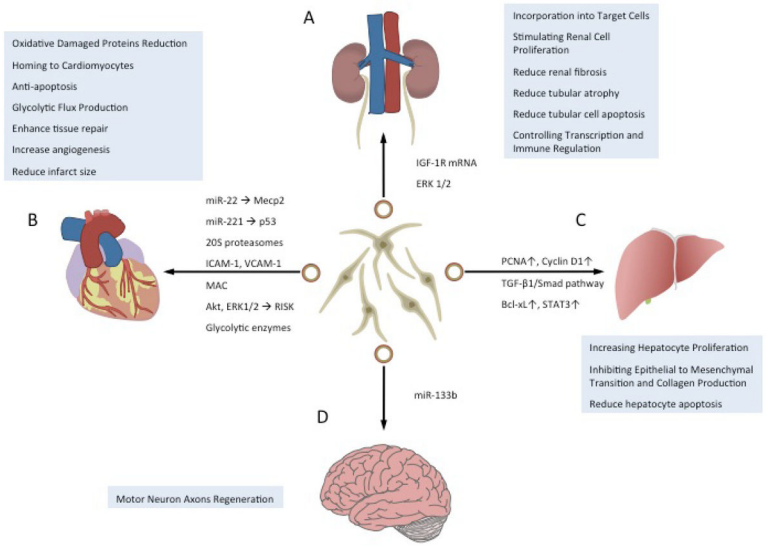

Figure 3. Therapeutic Properties of Extracellular Vesicles Derived From Mesenchymal Stem Cells in Various Organ Injuries

A) Acute Kidney Injury: MSC EV provided reno-protection by horizontal transfer of IGF-1R mRNA to renal tubular cells and by activating ERK½ MAPK; **B**) Myocardial Infarction: MSC EV contained: 1) Integrins that could home exosomes to cardiomyocytes that expressed ICAM-1, a ligand for integrins, or to VCAM-1 on endothelial cells; 2) CD73, present on the surface of exosomes, activated reperfusion injury salvage kinases by increased expression of pro-survival protein kinases such as Akt and ERK½; 3) CD59 (protectin), a widely expressed glycosylphosphatidylinositol-anchored membrane protein, prevented the formation of membrane attack complexes and inhibited complement-mediated lysis; 4) Glycolytic enzymes that could ameliorate energy deficit and potentially increase glycolytic flux and ATP production in the reperfused myocardium; 5) Active 20S proteasomes, which is responsible for the degradation of approximately 90% of all intracellular protein damaged by oxidation; 6) And microRNAs, such as the anti-apoptotic effect of miR-22, which directly targeted Mecp2 and reduced the expression of p53 upregulated modulator of apoptosis via miR-221; **C**) Liver Injury: MSC EV inhibited epithelial to mesenchymal transition and collagen production by suppressing the activation of TGF-β1/Smad signaling pathway. MSC EV administration was also associated with higher expression of proliferation proteins (PCNA and cyclin D1), the anti-apoptotic gene, Bcl-xL, and STAT3; **D**) Brain Injury: MSCs transferred to injured neural cells EV microRNAs, such as miR-133b, which were involved in regeneration of motor neuron axons.

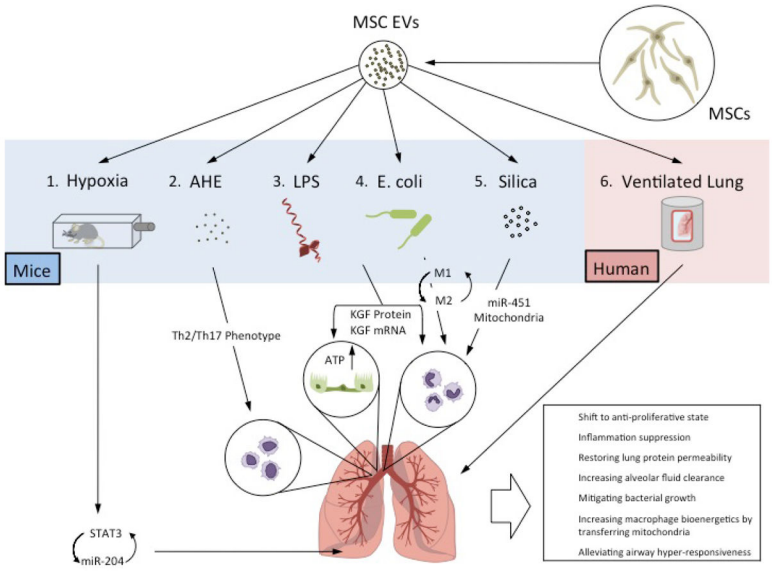

Figure 4. Therapeutic Properties of Extracellular Vesicles Derived from Mesenchymal Stem Cells in Lung Injury

1) In a mouse model of hypoxia-induced pulmonary artery hypertension, MSC EVs suppressed the hypoxic induction of STAT3 and up-regulated miR-204 levels, interfering with the STAT3-miR-204-STAT3 feed-forward loop and shifting the balance to an anti-proliferative state; **2**) In a mouse model of aspergillus hyphal extract-induced asthma, MSC EVs mitigated Th2/Th17-mediated airway hyper-responsiveness by shifting the Th2/Th17 inflammatory response towards a counter-regulatory Th1 response; **3)** In a mouse model of endotoxin-induced ALI, MSC EVs suppressed inflammation and restored lung protein permeability by transferring KGF mRNA to the injured alveolus, which restored both vectorial ion and fluid transport; **4**) In a mouse model of *Escherichia coli* pneumonia, MSC EVs reduced inflammation, lung protein permeability and pulmonary edema by decreased bacterial counts in the injured alveolus, leading to improved survival. MSC EVs were also found to enhance monocyte phagocytosis of bacteria, restore intracellular ATP levels in injured human alveolar epithelial type 2 cells, and repolarized monocytes/macrophages from a M1 to a M2 phenotype by possible transfer COX2 mRNA with subsequent secretion of PGE_2; **5)** In silica-induced ALI in mice, MSC-derived exosomes modulated toll-like receptor (TLR) signaling and cytokine secretion in macrophages, in part, by transfer of regulatory microRNAs such as mir-451 and prevented the recruitment of $Ly6^{Chi}$ monocytes and reduced secretion of pro-fibrotic IL-10 and TGFβ by these cells in the lung. In addition, MSCs managed intracellular oxidative stress by the extracellular transfer of depolarized mitochondria in vesicles to macrophages, improving bioenergetics; **6**) And in an *ex vivo* lung perfusion model of ischemia/reperfusion injury, restoration of alveolar fluid clearance by MSC EV was dependent on the internalization of EV into the injured host cells via CD44.