

Extracellular Vesicles Derived from Mesenchymal Stromal Cells: A Multidisciplinary Analysis of Methodology, Efficacy and Biodistribution in Lung Disease

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Abstract

Acute respiratory distress syndrome (ARDS) is a devastating critical illness that holds the highest cost of any acute care condition in Canada. In ARDS, a localized inflammatory response caused by lung infection or injury leads to vascular damage and migration of inflammatory cells into the airways resulting in impaired gas exchange and respiratory failure. Despite decades of research, there are no curative therapies for this daunting acute lung condition. Animal studies have demonstrated a potential for mesenchymal stromal cells (MSCs) to reduce inflammation, promote tissue regeneration and improve survival in acute lung injury (ALI, the preclinical correlate of ARDS). In particular, a growing body of evidence suggests that MSCs elicit their protective effects in large part by secretion of small membrane-bound particles known as “extracellular vesicles”. These vesicles carry biologically active cargo that modulate critical cell processes including programmed cell death, proliferation and inflammation. Hence, MSC-derived extracellular vesicles (MSC-EVs) may have advantages as a safer off-the-shelf, cell-free immune modulatory therapy. However, the optimal approach for isolating and administering MSC-EVs have yet to be established within this new field of regenerative medicine. The aims of this project were to: (1) evaluate the current preclinical evidence on methodology and efficacy of MSC-EVs, (2) determine the effectiveness of MSC-EVs for ALI and other pulmonary diseases in animal models, and (3) explore the kinetics and biodistribution of MSC-EVs during healthy and disease state as a prerequisite to designing optimal therapeutic strategies. By combining both clinical meta-research methodology and basic science techniques, I hoped to create a comprehensive evidence map of mechanistic insights and the therapeutic benefits of MSC-EVs.

Importantly, ARDS is recognized as the main cause of mortality for patients with COVID-19. In response to the SARS-CoV2 pandemic and restrictions placed on routine, non-COVID

related lab work, an additional objective for my PhD focused on developing a novel, more accessible mouse model of COVID-19 associated ARDS. A SARS-CoV2 “pseudovirus” was created to enable its use in a less stringent Containment Level 2 lab setting. Dose-escalation experiments in mice helped determine the optimal dose that produces clinically-relevant COVID-19 ARDS pathology. Moreover, the specificity of our pseudovirus to human ACE2 receptors was tested using preclinical models. If successful, this model will allow researchers worldwide to study key mechanisms in COVID-19 pathophysiology and investigate novel vaccines or therapies to combat this global pandemic.

*“Human knowledge is never contained in one person.
It grows from the relationships we create between each other and the world,
and still, it is never complete.”*

–Dr. Paul Kalanithi, When Breath Becomes Air

This dissertation is dedicated to my family, friends, and mentors.

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List of Abbreviations

ACE2	Angiotensin Converting Enzyme 2
ALI	Acute Lung Injury
Ang-1	Angiopoietin-1
ANOVA	Analysis of Variance
ARDS	Acute Respiratory Distress Syndrome
ARRIVE	Animal Research: Reporting In Vivo Experiments
BALF	Bronchoalveolar Lavage Fluid
BCA	Bicinchonic Acid
BM-MSCs	Bone Marrow Derived Mesenchymal Stromal Cells
BPD	Bronchopulmonary Dysplasia
BSL	Biosafety Level
CCL2	Chemokine Ligand 2
CI	Confidence Interval
CIHR	Canadian Institutes of Health Research
COPD	Chronic Obstructive Pulmonary Disease
COVID-19	Coronavirus Disease of 2019
CXCR4	C-X-C Motif Chemokine Receptor 4
DiR	1,1'-Diocadecyl-3,3',3'-Tetramethylindotricarbocyanine Iodide
DMBT1	Deleted In Malignant Brain Tumors 1
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
E. Coli	Escherichia Coli
ELISA	Enzyme-Linked Immunosorbent Assay
EMMPRIN	Extracellular Matrix Metalloproteinase Inducer
EV	Extracellular Vesicle
EV-TRACK	Transparent Reporting And Centralizing Knowledge in Extracellular Vesicle Research
FBS	Fetal Bovine Serum
FEV1	Forced Expiratory Volume in 1 Second
FGF	Fibroblast Growth Factor
FiO2	Fraction of Inspired Oxygen
hACE2	Human Angiotensin Converting Enzyme 2

HEK293T Cells	Human Embryonic Kidney 293 T Cells
HGF	Hepatocyte Growth Factor
HIF1 α	Hypoxia Inducible Factor 1 Alpha
HLA-DR	Human Leukocyte Antigen, DR Isotype
HSP	Heat Shock Protein
I.T.	Intratracheal
I.V.	Intravenous
ICU	Intensive Care Unit
IDO	Indolamine 2,3 Dioxygenase
IFN γ	Interferon Gamma
IL-1 β	Interleukin-1beta
IL-6	Interleukin-6
iPSCs	Induced Pluripotent Stem Cells
ISCT	International Society for Cellular Therapy
ISEV	International Society for Extracellular Vesicles
KGF	Keratinocyte Growth Factor
LPS	Lipopolysaccharide
LUNG SAFE	Large Observational Study to Understand the Global Impact of Severe Acute Respiratory Failure
MEM- α	Minimum Essential Medium Alpha
MERS	Middle East Respiratory Syndrome
MISEV2018	Minimal Information for Studies of Extracellular Vesicles 2018
mRNA	Messenger Ribonucleic Acid
MSC	Mesenchymal Stromal Cell
MSC-EVs	Mesenchymal Stromal Cell-Derived Extracellular Vesicles
MVB	Multivesicular Body
NF-kB	Nuclear Factor-Kappa-B
NIH	National Institute of Health
NTA	Nanoparticle Tracking Analysis
PAH	Pulmonary Arterial Hypertension
PaO ₂	Partial Pressure of Oxygen
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEEP	Positive End-Expiratory Pressure

PEG	Polyethylene Glycol
PFU	Plaque-Forming Unit
PGE2	Prostaglandin E2
PICOS	Population, Intervention, Comparator, Outcome and Study Design
Poly I:C	Polyinosinic:Polycytidylic Acid
PRESS	Peer Review of Electronic Search Strategies
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PROSEVA	Proning Severe ARDS Patients
RAAS	Renin-Angiotensin-Aldosterone System
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RR	Risk Ratio
RVSP	Right Ventricular Systolic Pressure
SARS-CoV2	Severe Acute Respiratory Syndrome Coronavirus 2
SEC	Size Exclusion Chromatography
SEM	Scanning Electron Microscopy
siRNA	Small Interfering Ribonucleic Acid
SMD	Standardized Mean Differences
SP1	Specificity Protein 1
SYRCLE	Systematic Review Centre for Laboratory Animal Experimentation
TEM	Transmission Electron Microscopy
TFF	Tangential Flow Filtration
TGFβ	Transforming Growth Factor Beta
TLR4	Toll-Like Receptor 4
TMPRSS2	Transmembrane Protease Serine 2
TNFα	Tumor Necrosis Factor Alpha
TSG101	Tumor Susceptibility Gene 101
UC-MSCs	Umbilical Cord Derived Mesenchymal Stromal Cells
VEGF	Vascular Endothelial Growth Factor
VSV	Vesicular Stomatitis Virus
VSV-CoV2-S	Vesicular Stomatitis Virus Bearing the SARS-CoV2 Spike Protein
WHO	World Health Organization

Chapter 1 – Introduction

Chapter 1 – Introduction Co-Authorship Statement

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1. Allan D, **Tieu A**, Lalu M and Burger D (2020). Mesenchymal stromal cell-derived extracellular vesicles for regenerative therapy and immune modulation: Progress and challenges toward clinical application. *Stem Cells Translational Medicine*, 9(1), pp.39-46

Author Contributions:

AT: Contributed to conceptualization of subjects to be included in review article. Wrote and revised multiple sections of the review article.

DSA: Primary author of review article.

ML and DB: Provided expertise in content and assisted in revising manuscript.

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AT: Wrote the editorial, generated the figure, and addressed revisions.

SDJ and ML: Provided expertise in content of editorial and assisted in writing/revising manuscript.

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Author Contributions:

AT: Created the systematic review protocol including the research question and methodological approach. Wrote the manuscript and generated the figures/tables.

MML and DSA: Principal investigators of project. Contributed to writing of manuscript, creation of protocol, and finalizing research question and methodological approach.

MS, DAF, JM, DB and DJS: Assisted in developing systematic review protocol and review of manuscript.

RS: Created search strategy of systematic review.

1.1 Acute Respiratory Distress Syndrome

It has been over fifty years since Dr. David Ashbaugh published the first description of the life-threatening illness known as acute respiratory distress syndrome (ARDS).¹ Twelve patients were admitted to the intensive care unit (ICU) for management of respiratory distress caused by a variety of precipitating factors such as trauma, viral pneumonia or acute pancreatitis. Despite unrelated physical and/or biochemical aetiologies, all patients exhibited remarkably similar clinical presentations of lung injury including severe dyspnea, tachypnea and hypoxemia necessitating mechanical ventilation. Moreover, a loss of lung compliance was consistently observed between patients. On chest x-ray, diffuse alveolar infiltrates and lung consolidation indicative of pulmonary edema was confirmed. Histological analysis identified the presence of hyaline membranes in nearly all patients, which is considered a hallmark finding for ARDS to this day. Despite decades since this initial depiction by Dr. Ashbaugh, many of the central characteristics that were described remain fundamental to our current understanding of ARDS.

In 2012, the ARDS Definition Task Force coined the Berlin Definition as a set of new diagnostic criteria for ARDS based on the most current epidemiological, physiological and clinical trial data.² From the Berlin Definition, ARDS can be categorized into three levels of severity depending on degree of hypoxemia (arterial partial pressure of oxygen (PaO₂) to fraction of inspired oxygen (FiO₂), P/F ratio): (1) mild (P/F ratio of 201–300 mmHg), (2) moderate (P/F ratio of 101–200 mmHg), and (3) severe (P/F ratio $\leq$ 100 mmHg).² In addition, this condition is characterized by three supplementary clinical features: *Imaging* (bilateral infiltrates consistent with pulmonary edema), *timing* (acute onset of less than 7 days from insult), and *origin* (not caused by cardiac failure or volume overload). Due to the complexity in ARDS pathophysiology, additional non-diagnostic parameters that may support a diagnosis of ARDS include testing for

pulmonary compliance or biomarkers like plasma angiopoietin 2 for lung vascular permeability and cytokines (interleukin-6 (IL-6), IL-1 β , IL-8) as inflammatory indicators.^{3,4}

1.2 Epidemiology of ARDS

A prospective cohort study of 21 hospitals in King County, Washington was conducted between 1999-2000 to estimate epidemiology data for ARDS.⁵ Their findings suggested an incidence of nearly 200,000 ARDS cases per year in the United States, which was associated with 3.6 million hospital days and a mortality rate of 40%.⁵ More recently, the Large Observational Study to Understand the Global Impact of Severe Acute Respiratory Failure (LUNG SAFE) was conducted as a 50-country multicenter trial, enrolling 29,144 ICU patients.⁶ LUNG SAFE found that 3022 patients (10.4%) fulfilled ARDS criteria, and 23% of patients required mechanical ventilation. Mortality rates varied depending on severity of hypoxemia (35% for mild ARDS (95% CI, 31-38%), 40% for moderate ARDS (95% CI, 38-42%), and 46.1% for patients with severe ARDS (95% CI, 42-50%)). Nonetheless, there is a high risk for death in patients diagnosed with any degree of ARDS worldwide.

There are limitations to epidemiologic studies of ARDS that are worth considering. First, ARDS remains underdiagnosed and undertreated globally.⁴ Despite international efforts to standardize diagnosis (e.g. the Berlin Definition), the LUNG SAFE study found that physician recognition of ARDS is only 51% in patients presenting with mild ARDS, and 79% in those with severe ARDS.⁶ Among patients that were correctly admitted for ARDS, more than one third of patients did not receive optimal tidal volume during protective mechanical ventilation.⁶ Possible explanations for these underestimates include inaccuracy in coding of diagnoses, incomplete data to diagnose ARDS prior to death, and most importantly, absence of any specific diagnostic test for

ARDS due to the wide range of causes.⁷ Moreover, the Berlin criteria is complicated by two issues: (1) Chest radiographs are crucial to attribute edema to pulmonary causes, but interpretation is subjective and unreliable; and (2) assessment of hypoxemia depends on treatment status and time of measurement.⁷ For example, patients who are already on positive end-expiratory pressure (PEEP) and oxygen therapy may no longer present with P/F ratios that correspond to an ARDS diagnosis.

1.3 Causes and Risk Factors of ARDS

Bacterial and viral pneumonia are the most prevalent causes of ARDS.^{4,8,9} Other common inciting factors include pulmonary aspiration, sepsis due to non-respiratory causes, and major trauma (e.g. blunt force or penetrating lung injury, severe burns). Although infrequent, ARDS can also be elicited by transfusion of blood products, inhalation of noxious substances, acute pancreatitis, or drug overdose.^{4,5} There is ongoing research attempting to identify environmental exposures, genetic factors and comorbidities associated with increased susceptibility for ARDS. Chronic alcohol abuse,¹⁰ cigarette smoking,¹¹ high body mass index,^{12,13} and environmental pollutants^{14,15} have all been demonstrated to be independently associated with the development of ARDS.

Despite efforts with genome-wide association studies, no genetic loci have been identified, in part due to the disparate causes of ARDS. However, there are candidate genes that may influence the progression and outcomes in patients with ARDS by encoding for important proteins that regulate pulmonary inflammation (e.g. IL-10, IL-6), vascular function (e.g. vascular endothelial growth factor), coagulation (e.g. protein C, plasminogen activator inhibitor 1) and alveolar-capillary barrier (e.g. angiopoietin 2).^{4,9,16} In association studies investigating ethnic differences in

ARDS outcomes, African American and Hispanic patients have been found to have a significantly elevated risk for mortality as compared to Caucasians,¹⁷⁻¹⁹ which may be attributed to a greater severity of illness on initial presentation.¹⁷ However, this finding is inconsistent as separate studies have reported lower incidences of ARDS in African American patients.²⁰ Accordingly, conflicting findings also exist regarding sex differences for ARDS related mortality as data from the LUNG SAFE study suggests females have a higher risk of death,²¹ whereas other reports found mortality to be elevated for male patients.^{18,19} Unfortunately, a physiological mechanism to explain ethnic and sex disparities has yet to be discovered.

1.4 Pathophysiology of ARDS

Alveoli in healthy lungs facilitate the removal of carbon dioxide and uptake of oxygen from the blood, allowing for delivery of oxygen to all tissues in the body. This gas exchange occurs across the alveolar-capillary barrier that is defined by a single layer of endothelial cells, a thin layer of interstitial space, and the epithelial layer within the alveoli. Alveolar septa consist of basement membrane, collagen and elastic fibers that separate adjacent alveoli. There are three main cells that makeup the inner alveolar lining: (1) type I pneumocytes that are thin and flat, forming the main alveolar structure, (2) type II pneumocytes that secrete pulmonary surfactant to reduce surface tension and prevent lung collapse, as well as differentiate into type I pneumocytes in the setting of lung injury, and (3) alveolar macrophages which serve as immune cells targeting foreign substances and pathogens by phagocytosis.

During ARDS, a localized inflammatory response, accumulation of leukocytes and platelets, dysregulated coagulation, and hyperpermeability caused by respiratory insult leads to impaired gas exchange (**Figure 1.1**).^{22,23} There are three distinct phases of disease progression.

The initial *exudative phase* commences within the first 24 hours after pulmonary injury and is characterized by diffuse alveolar damage.^{4,16} Alveolar macrophages respond to pathogen-associated molecular patterns (e.g. lipopolysaccharides, which are bacterial endotoxins found on gram-negative bacteria) or damage-associated molecular patterns (e.g. endogenous signals of tissue injury) through pattern recognition receptors. Activation of signaling cascades polarize macrophages to a pro-inflammatory phenotype releasing a myriad of inflammatory mediators (e.g. IL-6, IL-1 β , tumor necrosis factor (TNF)) and chemokines (e.g. IL-8, chemokine ligand 2 (CCL2)) that recruit innate immune cells. Circulating neutrophils and monocytes extravasate from systemic circulation to the site of lung injury. The concerted inflammatory response results in further insult to the integrity of the alveolar-capillary barrier, promoting interstitial and intra-alveolar accumulation of protein-rich fluid, inflammatory cells, and red blood cells (**Figure 1.1**). In parallel, microvascular injury and endothelial activation results in secretion of tissue factor to trigger excessive coagulation, platelet aggregation and thrombi formation. The exudative phase leads to compromised gas exchange and ultimately induces hypoxemia during ARDS.

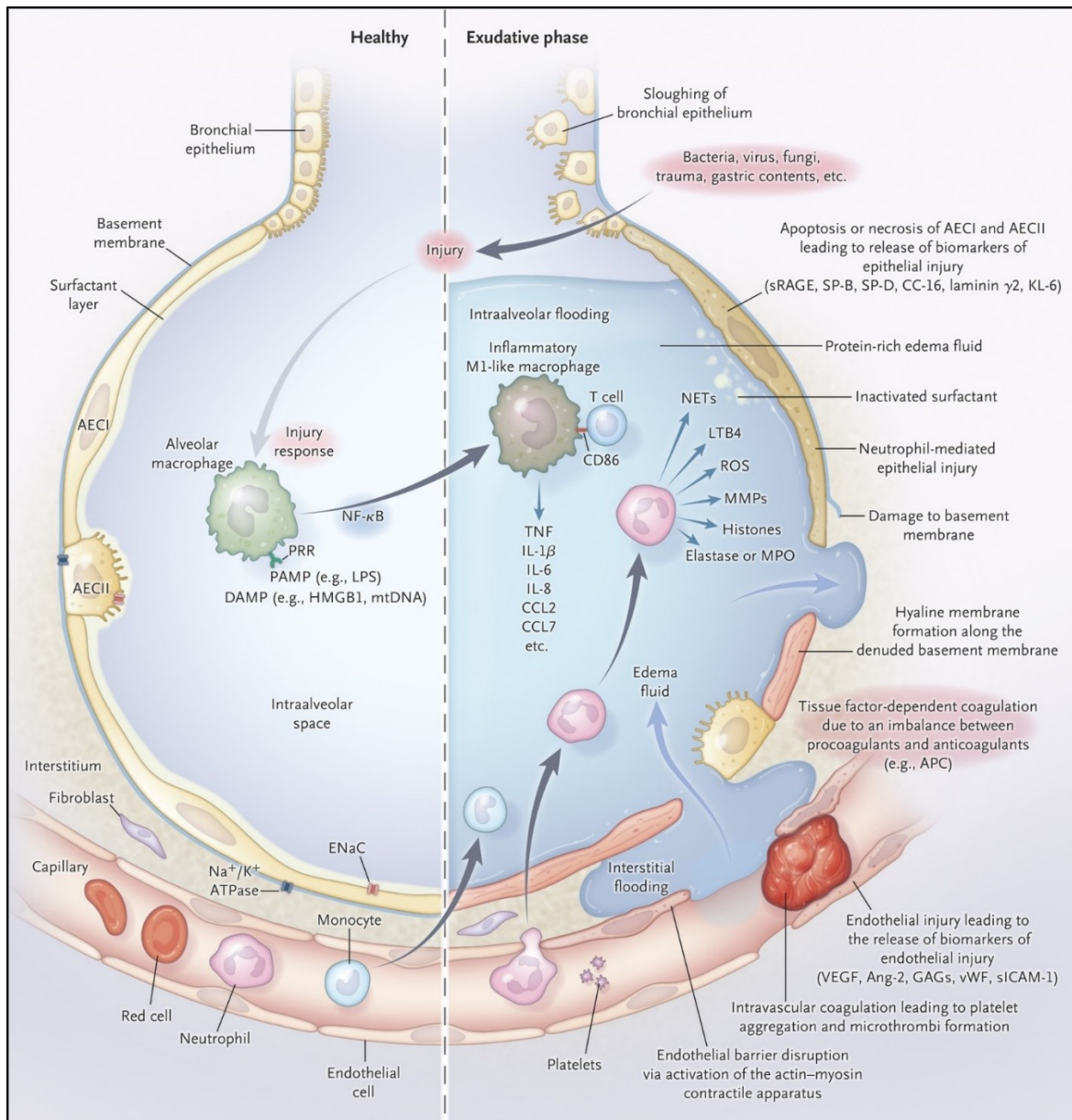


Figure 1.1. Pathophysiology of ARDS.

Comparison of healthy lung alveolus to the exudative phase of ARDS. During the exudative phase, inflammation in response to lung injury results in recruitment of neutrophils and activation of pulmonary macrophages. Subsequent production of pro-inflammatory cytokines contributes to disruption of the alveolar-capillary membrane barrier and reduces gas exchange. Moreover, endothelial injury causes dysregulated coagulation and microthrombi formation. Image adapted from Thompson *et al.* (2017).¹⁶

The second *proliferative phase* of ARDS ensues 7-14 days after the initial lung injury and is essential to patient recovery.¹⁶ As the name entails, this stage is characterized by extensive proliferation and restructuring of cells within the damaged alveoli. Type II pneumocytes differentiate into type I pneumocytes to reform the epithelial layer along the denuded basement membrane. Enhanced expression of ion channels and aquaporins allow for removal of intra-alveolar fluid to reduce pulmonary edema.²⁴ Endothelial progenitor cells promote repair of the endothelial barrier to re-establish gas exchange.²⁵ Clinically, patients may begin to exhibit signs of improvement with a reduced need for supportive care.

The final *fibrotic phase* of ARDS may occur in some patients and is characterized by the obliteration of normal pulmonary architecture.¹⁶ If the alveolar basement membrane is severely injured, adequate repair and regeneration may not be feasible. Instead, extensive proliferation of fibroblasts and myofibroblasts result in fibrotic lung tissue. Of note, ventilator-associated lung injury can contribute to this fibrogenic response.²⁶ Furthermore, patients that do progress into the fibrotic phase are associated with reduced ventilator-free days and increased mortality.²⁷

1.5 Management of ARDS

Despite decades of research, treatment of ARDS is limited to supportive care with no effective specific therapy. Optimal management requires early diagnosis, determining and treating the inciting cause, providing adequate respiratory support, and maintaining a balance of fluid/nutritional intake. Understanding the pathophysiology of ARDS has also allowed us to measure select biomarkers as a supplemental evaluation of disease severity. These include markers of epithelial injury (surfactant protein D, club cell protein 16), lung vascular permeability (von Willebrand factor, angiopoietin 2), dysregulated inflammation (IL-6, IL-8, TNF α), and activated

coagulation (protein C, plasminogen activator inhibitor 1).⁴ Based on secondary analyses of clinical trials, it has been proposed that patients with ARDS can be classified into hyper-inflammatory or hypo-inflammatory responders based on levels of plasma biomarkers, which has potential to guide differential treatment approaches and predict clinical outcomes.²⁸⁻³¹

1.5.1 Mechanical Ventilation

Lung-protective mechanical ventilation is foundational to treating patients with ARDS. Historically, mechanical ventilation was conducted using high tidal volumes and elevated plateau pressures.¹⁶ However, preclinical *in vivo* studies and clinical trials found that excessive mechanical stress from elevated tidal volumes and pressures further exacerbates pulmonary inflammation, resulting in ventilator-associated lung injury.^{9,32} A landmark trial published in 2000 demonstrated that reducing tidal volumes during mechanical ventilation results in significant improvements to survival, increases ventilator-free days, attenuates plasma IL-6 concentration and accelerates patient recovery.³³ Since then, controlled ventilation with a tidal volume of 4–8 mL/kg and inspiratory pressures ≤ 30 cm H₂O have been endorsed internationally for the treatment of ARDS.³⁴ A PEEP of 5–20 cm H₂O is currently recommended to improve alveolar recruitment and minimize the cyclical opening and collapse of distal alveoli.^{4,16,35} Importantly, maintaining high PEEP may increase the chance of survival for patients with moderate to severe ARDS.^{34,36}

1.5.2 Prone Positioning

In supine position, the lungs are compressed by the heart and abdominal organs, which inhibits full expansion and increases the risk for atelectasis.³⁷ In the prone position, redistribution of the blood and air flow enables homogenous lung inflation to improve blood oxygenation.^{35,37} With less support required from mechanical ventilation, the incidence of ventilator-associated lung injury is attenuated. In the PROSEVA clinical trial, prone positioning of patients with moderate to

severe ARDS for at least 16 hours dramatically reduced mortality.³⁸ Hence, prolonged prone positioning is now recommended within international guidelines for treating ARDS.^{34,39}

1.5.3 Pharmacological Therapies

Although our physiological understanding of ARDS has progressed significantly over the last 50 years, there are still no pharmacological therapies with proven survival benefits. Theoretically, the anti-inflammatory effects of glucocorticoids can reduce lung vascular permeability and injury. However, a multicenter trial of methylprednisolone found no improvements to survival and, conversely, increased risk of death during ARDS.⁴⁰ Moreover, glucocorticoids have also been associated with hypernatremia, hyperglycemia and neuromuscular weakness, which are crucial considerations prior to its use in patients.³⁵

Patient-ventilator asynchrony occurs when there is spontaneous breathing during mechanical ventilation. In this scenario, patients may suffer from cyclic atelectasis and shearing damage to the lung parenchyma.⁴ Sedatives and neuromuscular blockade can prevent mechanical injury by inducing paralysis. However, findings from clinical trials have been conflicting and no strong recommendations exist on neuromuscular blockade use for ARDS.^{41,42}

Other pharmacologic therapies have also been investigated but a paucity of robust evidence prevents their definitive recommendation. Inhaled nitric oxide and prostaglandins enable pulmonary vasodilation that can briefly improve blood oxygenation.⁴³⁻⁴⁵ A fluid-conservative approach has been suggested to reduce pulmonary edema, attenuate the need for mechanical ventilation and improve overall pulmonary function.⁴⁶ But similar to other therapies, no survival benefit from either approach is observed. Overall, novel interventions that can resolve the severe inflammation during ARDS is of critical importance to improve outcomes for this vulnerable

patient population. Testing of new therapies in relevant preclinical animal models can help elucidate the most promising treatments for clinical translation.

1.6 Experimental Models of ARDS

Animal models of acute lung injury (ALI, the preclinical correlate of ARDS) enable researchers to cultivate new mechanistic insights on the pathophysiology of ARDS, as well as test hypotheses generated from cellular/molecular experiments. Novel interventions can be evaluated for their therapeutic efficacy in small animal models prior to using larger animals that more closely represent the clinical disease. Ideally, animal models of ALI should replicate the pathogenesis and phenotypic findings that are observed in patients with ARDS. These features include acute onset, significant pulmonary inflammation, lung vascular hyperpermeability and diffuse alveolar damage. Although no single method of disease induction can reproduce all these characteristics, various animal models may offer advantages for specific, pertinent features of the disease.⁴⁷ Here, I will focus on reviewing the strengths and weaknesses on some of the more commonly utilized preclinical models of ALI.

1.6.1 Endotoxin-Induced ALI

Mechanism: Lipopolysaccharide (LPS) is a glycolipid endotoxin expressed on the outer membrane of Gram-negative bacteria. LPS binding proteins in plasma facilitate the interaction of LPS to toll like receptor 4 (TLR4), which is highly expressed on immune cells.⁴⁸ Subsequent intracellular signaling activates the nuclear factor-kB (NF-kB) pathway and upregulates the expression of multiple pro-inflammatory cytokines to induce inflammation and tissue injury.⁴⁸

LPS can be delivered intravenously or intratracheally to produce a model of ALI. Intravenous injection produces a systemic response attributed to widespread binding of LPS to the

endothelium.^{49,50} Animals present with fever, leukopenia, intravascular coagulation, and potentially multiorgan dysfunction.^{51,52} As LPS reacts with the pulmonary vasculature, neutrophil entrapment results in microvascular injury; however, intra-alveolar cell infiltration and changes to the alveolar membrane barrier may be delayed as significant vascular injury must first occur.⁴⁷

In models of intratracheal LPS, rapid binding of LPS to TLR4 on alveolar macrophages and epithelial cells produce an acute onset of lung injury. Notably, overwhelming neutrophils and macrophages accumulate within the alveolar air space, generating high levels of pro-inflammatory cytokines within the bronchoalveolar lavage fluid.⁵³⁻⁵⁶ As well, intratracheal LPS produces an earlier response in lung vascular hyperpermeability as compared to intravenous LPS. Histologically, diffuse alveolar damage, thickening of the alveolar septum, pneumocyte hyperplasia and occasional hyaline membranes can be observed.⁵⁷ These *in vivo* findings are clinically relevant features that are commonly observed in patients with ARDS.

Disadvantages and Considerations: Whether LPS is administered systemically or locally, there are species differences in response to LPS that need to be considered.⁴⁷ For example, rodents lack pulmonary intravascular macrophages and require larger doses of LPS for ALI induction. Manufacturing of LPS is another crucial consideration as specific endotoxin components (e.g. O-polysaccharide chain, lipid A, etc.) result in distinct responses to different animals.^{47,58} LPS preparation may also be contaminated with bacterial materials that alter biological effects.⁵⁹

Summary: Overall, LPS-induced ALI is one of the most commonly used animal models of lung injury. LPS allows for methodological simplicity, consistency and reproducibility. The injury produced is a direct consequence of the animal's inflammatory response to LPS binding, which accurately reflects a central component of ARDS in humans. Choice in route of administration is

dependent on the type of model desired. Intravenous LPS may result in a more systemic sepsis-like phenotype, whereas intratracheal LPS produces a more localized ALI response.

1.6.2 ALI Induction by Bacterial Inoculation

Similar to LPS, orotracheal administration of live bacteria can be used as a model of ALI. Delivery of bacteria can be achieved through an intratracheal catheter, surgical instillation or aerosolization within a chamber. *Escherichia coli*, *Streptococcus pneumoniae*, and *Pseudomonas aeruginosa* are some of the more commonly utilized bacteria for ALI induction.⁴⁷ The selected route of administration significantly influences infection response in animals. For example, intratracheal instillation produces localized pneumonia, whereas housing mice in an aerosol chamber enables widespread pulmonary infection in multiple mice simultaneously.⁶⁰ Nonetheless, delivery of live bacteria generates diffuse alveolitis, neutrophil accumulation into the airspace, elevation of inflammatory markers, microvascular permeability and pulmonary edema.^{47,57}

Disadvantages and Considerations: Intratracheal delivery enables a more precise infection of the lower respiratory tract; however, general anesthesia is required and consistency between animals and experiments is user dependent.⁶⁰ For aerosol administration, no anesthesia is necessary and less variability of infection may be observed between animals. Disadvantages include the expensive equipment that require regular maintenance, as well as limitations to choice of bacterial species as certain pathogens may not survive aerosolization or oxygen rich environments.⁶⁰ Other salient considerations that can affect disease outcomes include choice of bacteria, dose of inoculation and the immunoreactivity of the selected animal model.

Summary: ALI by live bacteria inoculation can be utilized as a model of pneumonia, the most common cause of ARDS in humans. The versatility in route of administration provides

researchers with the ability to achieve local or diffuse lung infections. However, careful attention to dosage regimen and bacterial species is needed to ensure adequate ALI induction.

1.6.3 Ventilator-Induced ALI

Mechanism: It is now well-recognized that patients on mechanical ventilation can suffer from ventilator-associated lung injury under high levels of tidal volume and plateau pressures. Interestingly, this practice changing theory stemmed in part from findings in early animal studies that reported significant pulmonary damage due to ventilator use.^{61,62} In this model, ALI is produced from mechanical pressure and alveolar overdistention.⁴⁷ The cellular response to mechanical stimuli involves synthesis and secretion of inflammatory cytokines, resulting in immune cell recruitment to the site of injury.⁶³⁻⁶⁵ The shear force produced by ventilators and excessive expansion of alveolar sacs cause separation of endothelial and epithelial cells from the basement membrane, resulting in hyperpermeability and pulmonary edema.^{66,67}

A notable advantage in ventilator-induced ALI includes the versatility in producing pulmonary damage in a relatively short period of time (<1 hour) or over longer durations (8+ hours) depending on the selected levels of tidal volume and airway pressure.^{47,66,68} The severity in ALI can also be adjusted whereby high tidal volumes result in more significant inflammation, pulmonary hemorrhage, hyaline membrane formation and altered lung compliance.⁴⁷

Disadvantages and Considerations: The main disadvantage for this model is the technical challenges associated with setting up the mechanical ventilation system and optimizing the approach for *in vivo* use, especially for small animal models.⁴⁷ Requirements of continuous anesthesia can lower body temperature, further contributing to respiratory failure. In addition, ventilation duration does not accurately reflect the clinical scenario as patients may require assisted respiration for days to weeks.

Summary: The ventilator-induced ALI exhibits characteristics that are similar to ARDS including lung epithelial and endothelial injury, alveolar edema, and marked inflammation.⁵⁷ This approach is clinically relevant as injury is a direct result of ventilator use (“ventilator-induced lung injury”); however, this does not accurately represent “ventilator-associated lung injury”, which occurs when mechanical ventilation augments respiratory injury after patients have been diagnosed with ARDS from a separate inciting cause.⁴⁷ Hence, the most common aetiologies of ARDS such as pneumonia, sepsis or trauma are not captured when utilizing this model of ALI.

1.6.4 Assessing Preclinical Outcomes of ALI

After selecting an *in vivo* model of ALI, researchers must assess preclinical outcomes that are relevant to the pathogenesis of ARDS. Unfortunately, ARDS is primarily diagnosed by clinical findings that are difficult to replicate in a preclinical setting. For one, radiographic imaging on small animals is expensive and not widely accessible. Physiological assessments of lung compliance or hypoxemia are impractical due to inconsistencies in technique and undesired consequences (e.g. ventilation-associated lung injury, trauma from arterial blood collection, reduced plasma volume for analysis). Hence, the American Thoracic Society organized an international committee of scientists to synthesize guidelines on the most relevant features of experimental ALI.^{57,69}

1) Histopathological Evidence of Lung Injury

Histological evidence of lung injury was determined to be the most clinically relevant finding in animal models of ALI. Lung sections should be prepared from paraffin-embedded tissues and stained with hematoxylin and eosin for qualitative and quantitative evaluation.⁵⁷ On gross examination of sections, obvious discoloration and diffuse alveolar damage (e.g. marked cellular infiltration, pneumocyte hyperplasia, pulmonary hemorrhage, interstitial thickening,

fibrosis) should be evident (**Figure 1.2**). For quantitative assessment, a standardized scoring system was created to mitigate variability between investigators.⁵⁷ Microscopic fields should be selected at random and evaluated in a blinded fashion to prevent bias. Five important histological findings are scored using a three-tiered system: (1) Intra-alveolar neutrophil accumulation, (2) neutrophil infiltration into the pulmonary interstitium, (3) alveolar septal thickening, (4) hyaline membrane formation, and (5) evidence of proteinaceous debris (e.g. fibrin strands) within alveoli.

2) Lung Vascular Hyperpermeability

Damage to the alveolar-capillary barrier is central to the development of ARDS. During the exudative phase, overwhelming inflammation causes microvascular injury and breakdown of the epithelium tight junctions enabling exudation of protein-rich fluid. Hence, total protein concentration in bronchoalveolar lavage fluid (BALF) is commonly measured to evaluate lung vascular hyperpermeability.⁵⁷ Alternatively, intravenous injection of Evans Blue can be used to assess the degree of lung staining, an indicator of heightened permeability.⁷⁰ A cruder appraisal of permeability is done by calculating the wet-to-dry ratio of lungs (e.g. wet lungs are weighed, dried in an incubator, and re-weighed; large ratios are consistent with significant injury). Lastly, intravenous injection of a labeled protein (e.g. radioactive or fluorescent albumin) offers a precise way to assess alveolar leakage by measuring their final concentration in BALF.⁵⁷

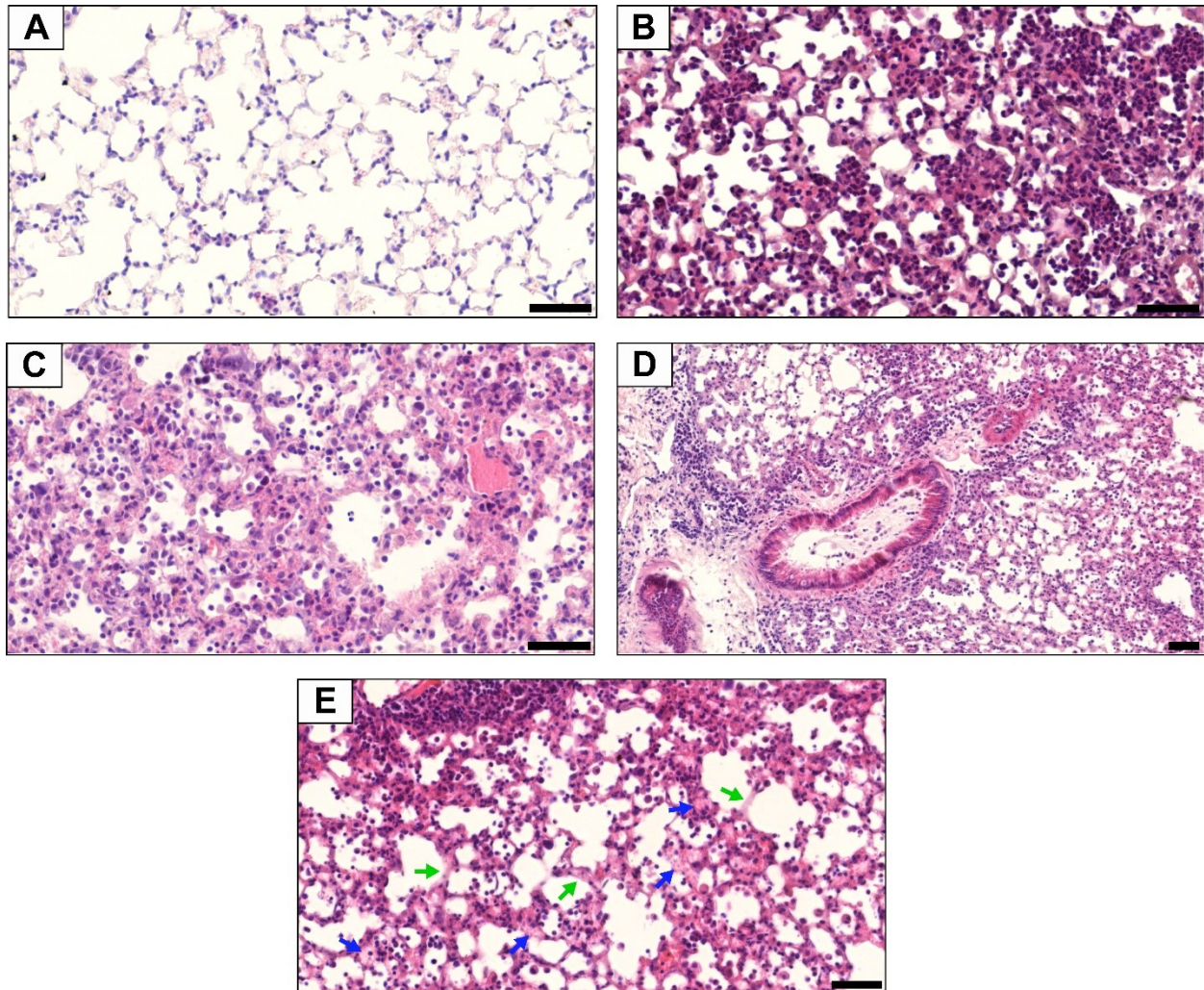


Figure 1.2. Histological images of ALI features.

(A) Intratracheal delivery of vehicle control (phosphate buffered saline) to wildtype mouse. (B) Lipopolysaccharide induced ALI in wildtype mouse with histological features including significant cellular infiltration, pneumocyte hyperplasia, alveolar septal thickening. (C–E) Viral induced ALI in mice highlighting additional features of (C) pulmonary hemorrhage and thrombus, (D) perivascular and peribronchiolar cellular infiltration, and (E) formation of hyaline membranes (green arrows) and fibrin strands (blue arrows). Scale = 50µm.

3) Measures of Inflammatory Response

One can measure cellular or molecular inflammatory changes in the lungs to determine the severity of ALI. Given the large role of innate immunity during ALI progression, elevated neutrophil count in BALF serves as a useful indicator for pulmonary inflammation. Cells can be pelleted onto microscope slides using a cytospin centrifuge.^{71,72} Percentage of neutrophils can be

calculated by light microscopy and the total number of neutrophils can be derived. Alternatively, flow cytometry can also be used to quantify BALF neutrophils.

Myeloperoxidase is a glycoprotein readily expressed by activated neutrophils and can be used as a surrogate marker for neutrophil count.^{73,74} Moreover, heightened expression of inflammatory cytokines (e.g. IL-6, IL-1 β , IL-8, TNF α , TGF β and many more) in BALF indicate severe lung inflammation and immune cell activation.

4) *Physiological Dysfunction*

The American Thoracic Society guidelines suggest that hypoxemia and alveolar-arterial oxygen gradient are relevant outcomes in preclinical ALI.⁵⁷ Unfortunately, these physiological changes of ARDS in humans (e.g. ventilation-perfusion mismatch, reduced lung compliance, severe hypoxemia) are difficult to assess *in vivo*. Multiple factors unrelated to ALI can cause extreme variability including animal stress, technician experience, choice of equipment or anesthetic, level of sedation and many others. The diagnostic criteria for P/F ratio and minute ventilation have also not been standardized across animal species. Given these inconsistencies, assessments of physiological dysfunction remain to be minor outcomes for *in vivo* ALI. Fortunately, investigators can be confident that a model of ALI was achieved if there is evidence of pronounced histological lung injury, vascular hyperpermeability and pulmonary inflammation.

1.7 Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV2)

The outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) has greatly impaired the progress of non-COVID-19 scientific research around the world. However, my thesis' emphasis on ARDS has enabled me to add a supplemental objective focused on COVID-19 associated ARDS to hopefully help advance our understanding of this novel infection.

From December 31, 2019 to January 3, 2020 in Wuhan, China, 44 patients were diagnosed with pneumonia of unknown origin.⁷⁵ Four days later, investigators were able to isolate and identify the new coronavirus now referred to as SARS-CoV2. On February 11, 2020, the World Health Organization (WHO) officially named the disease caused by SARS-CoV2 infection as COVID-19 (i.e. coronavirus disease 2019).⁷⁶ One month later, COVID-19 was declared a global pandemic.

Since the first reports of COVID-19, a meteoric rise in global infections has ensued. As of July 3, 2022, the coronavirus pandemic has resulted in nearly 550 million cases and over 6.3 million deaths worldwide.⁷⁷ SARS-CoV2 is a member of the coronavirus family alongside two other highly pathogenic viruses: (1) SARS-CoV that also originated from China, which was responsible for the 2003 SARS epidemic and (2) Middle East Respiratory Syndrome (MERS) CoV first identified in Saudi Arabia resulting in the 2012 MERS outbreak.⁷⁸ However, SARS-CoV2 has demonstrated greater infectivity due to the combination of having highly active viral replication in the upper respiratory tract, an earlier peak in viral load (e.g. within one week after symptom onset, compared to 10-14 days for SARS-CoV or 7-10 days for MERS-CoV) and prolonged viral shedding.⁷⁸⁻⁸² Moreover, the viral load in symptomatic and asymptomatic infected individuals have been found to be similar, which are contributing factors to the efficient spread of SARS-CoV2.⁷⁸ These characteristics of SARS-CoV2 point to the importance of early detection and expeditious isolation practices after findings of first symptoms.

The mode of transmission of SARS-CoV2 includes respiratory droplets and generated aerosols. There are three main mechanisms by which individuals can be exposed to the virus: (1) Inhalation of respiratory or airborne particles with the greatest risk within six feet of an infectious source, (2) deposition of viral particles onto mucous membranes including the eyes, mouth, or nose, and (3) contact with contaminated surfaces (fomites) followed by self-distribution of virus

onto mucous membranes.⁸³⁻⁸⁶ Given these various methods of viral spread, it is no surprise that there has been global uptake of precautions including mask mandates, physical distancing, shutdown of public spaces and international travel restrictions.

1.8 Clinical Features of COVID-19

The median incubation period of SARS-CoV2 infection is reported to be between 4-5 days, with a range from 2-14 days.⁸⁷⁻⁸⁹ Typical symptoms in the beginning stages of SARS-CoV2 infection present similarly to other viral pneumonia with fever, cough, shortness of breath, chills, sore throat and myalgias. As COVID-19 became more prevalent, some unique extrapulmonary features were discovered including conjunctivitis, chemosensory changes (e.g. anosmia or hyposmia, and hypogeusia, dysgeusia or ageusia), and gastrointestinal symptoms (e.g. diarrhea, abdominal pain).^{84,90} Patients with COVID-19 can be categorized into mild, moderate, severe or critical conditions.^{84,89} Mild or moderate illness (81% of patients) are accompanied by a broad range of nonspecific symptoms with negative findings of pneumonia and hypoxia.⁹¹ In patients with severe COVID-19 (14% of those infected), the presence of tachypnea, dyspnea, hypoxemia and/or radiological findings of respiratory infiltrates necessitates hospitalization.⁸⁴ Age is one of the primary predictors of complications as those 80 years or older have a mortality rate of 15% compared to <1% for patients younger than 40 years old.^{91,92} Comorbidities including obesity, cardiovascular disease, diabetes, chronic respiratory diseases, renal insufficiency and cancer have all been suggested to augment the risk of developing severe COVID-19.⁹²⁻¹⁰⁰

Critical illness is seen in 5% of patients and is characterized by potentially fatal complications including ARDS, shock and multiorgan failure requiring ICU management.^{84,91} ARDS remains to be the leading cause of death in patients with COVID-19 and the median time

from illness onset to diagnosis is 8-12 days.⁸⁹ The case fatality rate of patients with critical COVID-19 is high at 40-70%, highlighting its severity if left untreated.⁸⁹ Early on in the pandemic, studies indicated that COVID-19 associated ARDS may differ from classical ARDS as some patients presented with severe hypoxemia despite normal pulmonary compliance. If proven true, these concerns would have had profound implications on management of patients with critical SARS-CoV2 pulmonary infections.^{78,101-103} However, a prospective observational study comparing COVID-19 ARDS to classical ARDS found that typical functional and morphological features of reduced compliance and increased lung weight were exhibited by both patient populations.¹⁰⁴ Hence, authors concluded that the current evidence-based approach of lung-protective ventilation should continue to be used to avoid ventilator associated lung injury. Given the current uncertainty in our understanding of COVID-19 associated ARDS, it is clear that more robust evidence is needed before significant changes to management is warranted.

1.9 Extrapulmonary Manifestations of COVID-19

Beyond respiratory symptoms, it is becoming clear that SARS-CoV2 infection also induces a number of extrapulmonary manifestations.¹⁰⁵ A distinctive feature of COVID-19 is the associated coagulopathy observed in many patients. It is estimated that 20-70% of patients with critical COVID-19 develop arterial and/or venous thromboembolisms,^{106,107} which is drastically higher than the 8% observed in post-operative ICU patients.¹⁰⁸ Accordingly, patients with COVID-19 associated ARDS with low compliance and increased circulating levels of D-dimer were found to have a considerably higher risk of mortality.¹⁰⁴ Other hematologic responses in COVID-19 include lymphopenia (a hallmark finding of impaired immunity), leukocytosis, and neutrophilia.¹⁰⁵

Cardiovascular manifestations that have been associated with SARS-CoV2 infection are myocarditis, arrhythmias, cardiomyopathy and myocardial injury.¹⁰⁵ In the gastrointestinal system, COVID-19 produces a variety of non-fatal symptoms (e.g. anorexia, nausea, diarrhea), whereas neurological findings range from non-specific symptoms (e.g. headache, fatigue, anosmia) to severe presentations (e.g. impaired consciousness, ischemic stroke, encephalopathy, Guillain-Barre Syndrome). Although diabetic and obese patients are known to be at much greater risk for developing severe COVID-19, endocrinologic presentations including hyperglycemia and ketoacidosis have been observed in patients who have no underlying diabetes.¹⁰⁵

1.10 Pathophysiology of COVID-19

Early in the COVID-19 pandemic, SARS-CoV2 was discovered to be highly homologous with the original SARS-CoV.¹⁰⁹ Pivotal papers during the 2003 SARS-CoV epidemic identified human angiotensin converting enzyme 2 (ACE2) as the functional receptor for viral entry.^{110,111} Now, 19 years later, structural and functional analyses have validated that ACE2 is the same receptor involved in the mechanism for SARS-CoV2 cell entry and infection.¹¹²⁻¹¹⁴

SARS-CoV2 consists of four structural proteins: spike, envelope, membrane and nucleocapsid (**Figure 1.3**).¹¹⁵ The development of COVID-19 begins when inhalation of viral particles enables SARS-CoV2 invasion of epithelial cells in the upper respiratory tract. The spike protein of SARS-CoV2 has two subunits, S1 and S2, whereby S1 is responsible for binding to ACE2.¹¹⁵ Upon binding, transmembrane protease serine 2 (TMPRSS2), furin and other proteases expressed on the cell surface will cleave and activate the spike protein, allowing the S2 subunit to facilitate viral and host cell membrane fusion for endocytosis (**Figure 1.3**).^{113,116,117} The viral genome, a single stranded positive RNA, is translated by the host cell machinery to form the viral

replication and transcription complex.¹¹⁸ Subsequent replication and transcription of the SARS-CoV2 genome results in biosynthesis of viral proteins for assembly and secretion of new virions.

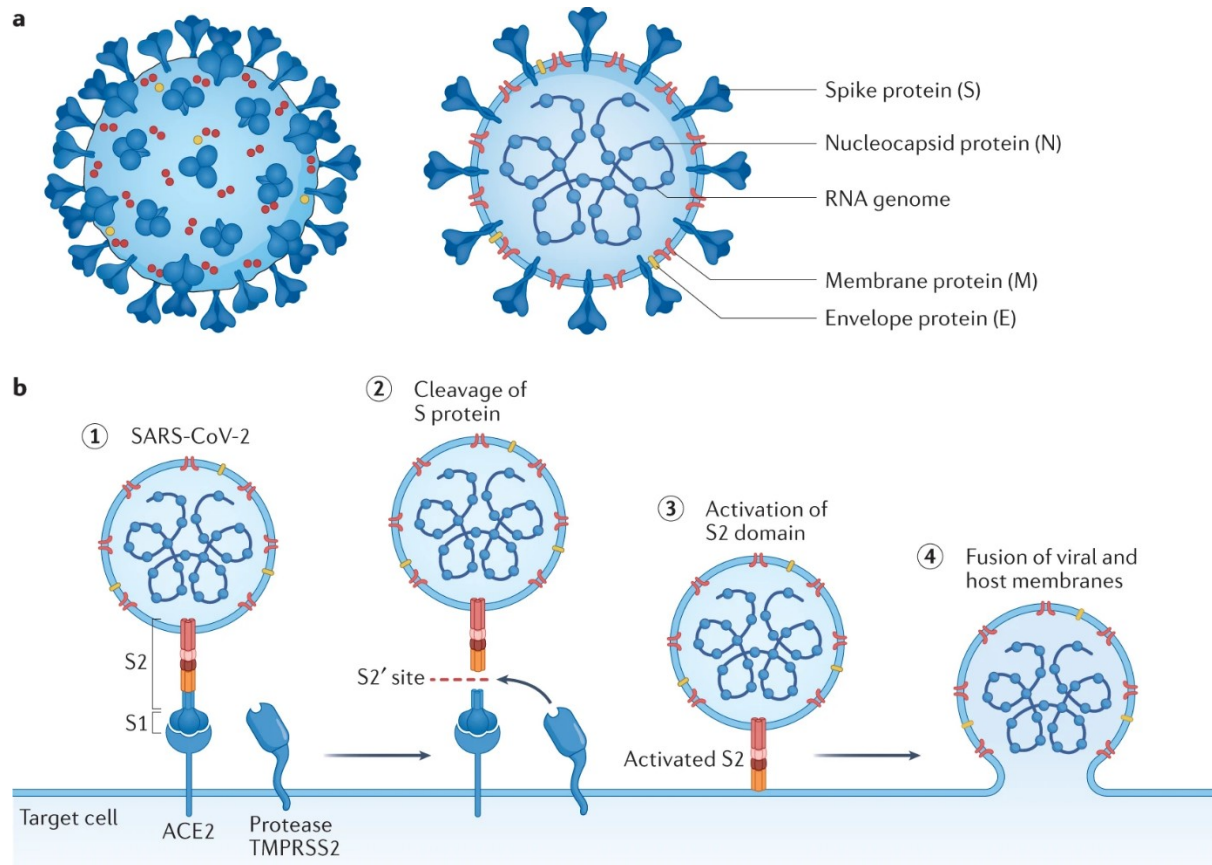


Figure 1.3. Pathophysiology of SARS-CoV2.

(A) Severe acute respiratory distress syndrome coronavirus 2 (SARS-CoV2) structure and components including spike, nucleocapsid, membrane and envelope proteins, as well as the positive single stranded RNA genome. (B) Binding of the SARS-CoV2 S1 subunit of spike protein to angiotensin converting enzyme 2 (ACE2) receptors on host target cells. Subsequent activation by transmembrane protease serine 2 (TMPRSS2) and other cell membrane proteases enables endocytosis of SARS-CoV2 for cell infection. Image adapted from Lamers and Haagmans (2022).¹¹⁹

Newly excreted SARS-CoV2 particles disseminate into the lower respiratory tract. Within alveoli, SARS-CoV2 targets pneumocytes and alveolar macrophages. The intracellular expression of viral pathogen-associated molecular patterns results in activation of inflammatory signaling cascades for production of type I interferons (IFNs), pro-inflammatory cytokines and chemokines.¹²⁰ Similar to the beginning stages of classical ARDS, recruitment of innate and adaptive immune cells augment the secretion of inflammatory mediators. If left unhampered, the continued cycling of viral replication and immune response can cause overwhelming inflammation and induce COVID-19 associated ARDS.

Mechanisms that have been shown to be prominent in COVID-19 include endothelial damage, dysregulation of the renin-angiotensin-aldosterone system (RAAS) and the extrapulmonary manifestations as a result of widespread SARS-CoV2 dissemination.¹⁰⁵ ACE2 expression on arterial and venous endothelium mediates endothelial injury, inflammation and activation.¹²¹ Physiologically, inappropriate thrombin synthesis, hindered fibrinolysis and initiation of complement pathways during COVID-19 causes microthrombi deposition and vascular thromboembolism.¹⁰⁵ As ACE2 is occupied by SARS-CoV2, an imbalance in RAAS is also observed resulting in impaired conversion of both angiotensin I and II to anti-inflammatory by-products.¹²² Rather, circulation of angiotensin I and II exacerbates inflammation and promotes systemic vasoconstriction. Lastly, ACE2 is shown to be widely expressed across different organ tissues, thereby providing a possible explanation for the diverse symptoms of COVID-19 outside of lung injury.^{123,124}

1.11 Current Treatment Guidelines for COVID-19

As our knowledge of COVID-19 has advanced over the past three years, therapeutic interventions have aimed to both hamper progression and manage severe disease. The mainstay of prevention remains to be vaccinations, which demonstrated 95% protective efficacy when first introduced in 2020.¹²⁵ For patients with mild to moderate COVID-19 who do not require supplemental oxygen, current treatment guidelines recommend symptom management with no role for steroid or antiviral interventions unless they are at elevated risk for progression to severe disease (e.g. elderly population, immunocompromised).¹²⁶ In patients who are hospitalized due to COVID-19 and require minimal oxygenation, dexamethasone and remdesivir should be considered. As patients progress to more critical illness illustrated by increasing oxygen needs through high flow nasal cannula, non-invasive ventilation or mechanical ventilation, prompt initiation of dexamethasone with either baricitinib or tocilizumab is recommended.¹²⁶ Although both drugs are approved for treatment of rheumatoid arthritis, they have now been repurposed for COVID-19 as baricitinib inhibits the enzyme janus kinase (JAK) thereby preventing inflammatory signalling,¹²⁷⁻¹³⁰ and tocilizumab is a monoclonal antibody that antagonizes IL-6 binding to mitigate a severe proinflammatory response.¹³¹⁻¹³³ Importantly, as new variants of SARS-CoV2 have become dominant, the efficacy of vaccinations have declined.^{134,135} There remains a need to develop new therapies specific for COVID-19 that can achieve greater therapeutic efficacy in improving survival and reducing prolonged hospitalizations.

1.12 Mouse Models of COVID-19

To accelerate the development of novel interventions and vaccines for COVID-19, researchers require a relevant preclinical animal model that recapitulates the mechanism by which

SARS-CoV2 infection occurs.¹³⁶ Although large animal models and non-human primates may represent infections that more closely resemble the clinical disease, there are considerable limitations in cost, specialized housing/facilities, veterinary services, long reproductive cycles and genetic modifications. Moreover, large animals have shown to develop only mild SARS-CoV2 pulmonary infections.¹³⁷ Hence, small animal models provide a more widely accessible approach in studying the pathogenesis of COVID-19. In the setting of a pandemic, mouse models can also be rapidly developed and dispersed in a global effort to find innovative solutions. However, all animal models of COVID-19 using live SARS-CoV2 are limited by the need for Containment Level 3 facilities, which are costly and not widely available.¹³⁸ This section will focus on the current mouse models that have been applied in the combat against COVID-19.

Genetically Modified Mice

The main obstacle in using mouse models of COVID-19 is the lack of SARS-CoV2 binding to rodent ACE2. The rise of SARS-CoV in 2003 led to development of genetically modified mice that express the human ACE2 (hACE2) receptor under the keratin 18 (K18) promoter in epithelial cells.¹³⁹ Intranasal delivery of SARS-CoV2 to this model has shown varying degrees of severity ranging from moderate to lethal lung injury dependent on the administered viral dose. Specifically, 1×10^5 (also “1E5”) pfu of SARS-CoV2 results in 20-30% weight loss with 100% mortality by day 5 to 8 post inoculation.¹⁴⁰⁻¹⁴³ At lower doses, a less severe ALI response is induced with full recovery in some mice over time. As a model of COVID-19, K18-hACE2 transgenic mice demonstrate elevated viral loads in the lungs and high specificity of SARS-CoV2 binding to hACE2 as wildtype mice are consistently unaffected.^{142,143} A significant inflammatory response is generated with drastic increases in expression of chemokines and cytokines. In addition, this mouse model exhibits moderate viral dissemination to other organs similar to the human

disease.^{140,141} Notably, the neurotropism of SARS-CoV2 in K18-hACE2 transgenic mice has been suggested as a contributing factor to animal mortality.^{141,144}

Other genetically modified mouse models that have been used include hACE2 expression driven by the mouse ACE2 promoter or HFH4/FOXJ1 lung epithelial cells. In the former, a non-lethal lung injury response was exhibited from intranasal SARS-CoV2 with 8% weight loss and full recovery by 14 days.¹¹⁴ On histological examination, a moderate interstitial pneumonia was evident. No pathology or viral titers were found in other organs, demonstrating a lung-localized model of COVID-19 ARDS. SARS-CoV2 delivery to HFH4/FOXJ1 hACE2 transgenic mice resulted in 50% mortality by day 6 and produced leukocytic infiltration, extensive perivascular infiltration and pulmonary edema on lung histology.¹⁴⁵

Adenovirus Mouse Models

Adenovirus-mediated expression of hACE2 potentially offers a more widely applicable approach to investigate COVID-19. Transgenic mouse models require backcrossing of animals and validation of genotype that may make it difficult to meet global demands. By using an adenovirus system, hACE2 can be directly inserted into the mouse genome within the lungs. However, gene expression may only be transient. In this mouse model, a localized ALI is observed with high pulmonary titers of SARS-CoV2.¹⁴⁶ Importantly, the same viral dose (1E5 pfu) that resulted in overwhelming inflammation, severe lung damage and 100% mortality in transgenic mice induced minimal weight loss, minor histological changes and no deaths when using adenovirus mediated hACE2 expression.¹⁴⁷ These findings were confirmed in another study that directly compared SARS-CoV2 infection of K18-hACE2 mice and adenoviral hACE2 mice.¹⁴⁸

Mouse Adapted SARS-CoV2

Although SARS-CoV2 cannot bind to mouse ACE2, mouse adapted SARS-CoV2 were developed through targeted mutations or serial passaging of the virus *in vivo*. In one study, reverse genetics were applied to remodel the SARS-CoV2 spike protein and optimize its binding of mouse ACE2.¹⁴⁹ Although mice exhibited elevated viral titres in the lung and histological findings of interstitial pneumonia, alveolar damage and lymphocytic infiltration, no clinical signs of disease were detected. In contrast, authors demonstrated 40% mortality from SARS-CoV2 inoculation in HFH4-hACE2 transgenic mice with similar findings of ALI features as previously reported.

In another study, a mouse adapted SARS-CoV2 was produced by serial passaging of coronavirus in mice.¹⁵⁰ The resultant virus was highly pathogenic and exhibited a dose-dependent effect on mortality in wildtype mice. Physiological assessments demonstrated marked pulmonary dysfunction that was associated with significant lung congestion and histological injury. Aged mice were also considerably more susceptible to morbidity and mortality from mouse adapted SARS-CoV2 inoculation. Hence, serial passaging of SARS-CoV2 for natural adaptation may enable production of viruses that induce more severe ALI and clinically-relevant features of COVID-19. However, a significant disadvantage would be the inability to assess and compare SARS-CoV2 variants of concern. Transgenic mice may serve as better suited models to investigate the pathogenicity of viral variants as the human form of coronavirus evolves.

Pseudotyped SARS-CoV2 Mouse Model

Pseudotyped viruses are generated by expressing the surface glycoprotein of one virus (e.g. SARS-CoV2 spike protein) on the structural core of another virus (e.g. vesicular stomatitis virus).¹⁵¹ A significant advantage of this approach is the marked reduction in risk associated with studying highly lethal and/or infective viruses. The pathogenicity and efficacy of interventions against the pseudotyped virus can be investigated using less restrictive biosafety facilities, thereby

enabling greater accessibility for researchers around the world. During this pandemic, pseudoviruses of SARS-CoV2 have been often used *in vitro* to assess the ability of antibodies or sera (*i.e.* neutralizing antibody therapies or serum obtained after vaccine exposure) in attenuating viral infectivity through neutralization assays.¹⁵²⁻¹⁵⁷

Some groups have also delivered pseudoviruses to animals as a potential approach for modeling COVID-19 *in vivo*. In one study, a vesicular stomatitis virus backbone expressing the coronavirus spike protein was generated as a SARS-CoV2 pseudovirus,¹⁵⁸ whereas another study utilized a lentivirus system for pseudovirus production.¹⁵⁹ Both groups incorporated luciferase activity within their pseudotyped viruses to demonstrate pulmonary infection by bioluminescence analysis. However, neither study assessed pulmonary specific outcomes and there were no indications that delivery of their pseudoviruses in mice expressing hACE2 induced a severe lung injury response comparable to SARS-CoV2.^{158,159} Although this approach circumvents the necessity for Biosafety Level 3 facilities, a pseudovirus animal model that better represents the disease course and severity of SARS-CoV2 infections is still needed to retain clinical relevance.

1.13 Mesenchymal Stromal Cells (MSCs)

Despite decades of research investigating ARDS, there remains to be no specific curative therapy for this devastating illness. This fact has become more apparent during the SARS-CoV2 pandemic as patients continue to suffer from COVID-19 associated ARDS without available rescue treatments. In the world of regenerative medicine, mesenchymal stromal cells (MSCs) have shown preclinical promise as an immunomodulatory biotherapeutic and is currently being tested in clinical trials for its efficacy to treat classical and COVID-19 ARDS. This section will provide an overview on the isolation, characterization and therapeutic potential of MSCs.

1.13.1 Sources of MSCs

MSCs (also commonly referred to as multipotent stromal cells) are a heterogeneous population of cells first discovered by Dr. Alexander Friedenstein in the 1970s.¹⁶⁰⁻¹⁶² In these early studies, MSCs isolated from bone marrow demonstrated an ability for multipotent differentiation. Since these findings, MSCs have received considerable scientific attention across numerous disease domains for their potential as a regenerative cell therapy. We now know that MSCs can be isolated from multiple tissue sources including adipose tissue, umbilical cord, synovial membrane, skin, and dental pulp, and can even be derived from pluripotent stem cells.¹⁶³⁻¹⁶⁵ Although bone marrow is still the most frequently utilized source for MSC expansion, the extraction procedure is invasive and can be painful for donors. In comparison, adipose tissue and umbilical cords have become more popular in recent years due to their abundance since both are considered biohazardous waste.¹⁶⁶ Donor characteristics including age, sex and comorbidities are all factors that can produce variability in the therapeutic efficacy of bone marrow derived MSCs.¹⁶⁷⁻¹⁷⁰ In comparison, these parameters may be more consistent when utilizing umbilical cord tissue.¹⁷¹ Lastly, reports indicate MSCs from umbilical cord are produced at higher yields and greater proliferative capacity as compared to other cell sources, while maintaining MSC marker expression and differentiation ability.^{164,172} Thus, the distinct advantages of resource availability, non-invasive acquisition and efficient cell expansion from umbilical cord tissue supports its utility as an MSC source in interventional preclinical and clinical trials.

1.13.2 Characterization of MSCs

Interest in studying and developing MSCs have exceled since their first discovery. However, poor reporting of methodology and inconsistent approaches for isolating and characterizing MSCs in the 1990s and early 2000s hindered progress within the field. It became

increasingly difficult to compare findings between studies and develop a robust understanding of MSC efficacy needed for clinical translation. In response to these issues, the International Society for Cellular Therapy (ISCT) proposed a standard set of minimal criteria to define MSCs: (1) Adherence to plastic in standard culture conditions, (2) $\geq 95\%$ of the MSC population expresses specific surface antigens (CD105, CD73, CD90) with negative expression ($\leq 2\%$) of hematopoietic and endothelial markers (CD45, CD34, CD14/CD11b, CD79 α , CD19 and human leukocyte antigen DR isotype (HLA-DR)), and (3) multipotent *in vitro* differentiation ability into osteoblast, adipocyte and chondrocyte lineages.¹⁷³

Although MSC characterization is now more widely consistent, unsubstantiated MSC nomenclature is a problem that remains rampant despite attempts of standardization. In 2005, the ISCT noted that the use of “mesenchymal *stem* cells” should be reserved for studies that definitively prove their cells exhibit a capacity for *in vitro* and *in vivo* self-renewal with differentiation potential.¹⁷⁴ In contrast, the term “mesenchymal *stromal* cells” describes a broader population of cells associated with the therapeutic efficacy of MSCs due to immunomodulatory, tissue homing and secretory functions. Despite this distinction in terminology, there is still a significantly greater number of publications that employ the term “stem cells”, evident by 76,231 records on PubMed when “mesenchymal *stem* cells” is searched as compared to only 8,033 results for “mesenchymal *stromal* cells” (searched on January 4, 2021). Hence, the ISCT recently issued an updated position statement to encourage more stringent evaluation by funding agencies, scientific journals and conferences for studies utilizing the term “stem cells”.¹⁷⁵

1.13.3 Therapeutic Mechanism of MSCs

Thousands of preclinical animal studies across multiple different disciplines (e.g. cardiology, nephrology, orthopedics, neurology and many more) over the last twenty years have

supported the notion that MSCs confer therapeutic benefit for a variety of disease conditions.¹⁷⁶⁻
¹⁸⁰ When administered systemically, they migrate to sites of disease/injury to promote cell survival, attenuate inflammation and foster tissue repair.^{181,182} These pleiotropic effects have been shown to be facilitated by the immunoregulatory capacity of MSCs and through release of paracrine factors.

Immunomodulation by MSCs

MSCs are known to sense the inflammatory environment. They express Toll-like receptors on their cell surface that recognize the molecules associated with pathogens or from tissue injury.¹⁸³ Within the innate immune system, macrophages play a crucial role in initiating the inflammatory cascade. MSCs assist macrophages in mounting an early phase immune response by releasing chemoattractants to recruit neutrophils and lymphocytes to the site of injury or infection. As the level of pro-inflammatory cytokines increase, MSCs adopt an anti-inflammatory phenotype and secrete factors such as prostaglandin E2 (PGE2), indolamine 2,3 dioxygenase (IDO) and IL-10 to promote the polarization of macrophages towards an immunosuppressive phenotype (“M2”).¹⁸³ Subsequently, M2 macrophages produce even greater levels of anti-inflammatory soluble factors (IL-10, TGFβ) to dampen inflammation.¹⁸⁴

The adaptive immune response is dependent on antigen presenting cells to drive the production of CD4+ helper and CD8+ cytotoxic T-lymphocytes for pathogen clearance. MSCs regulate the balance between inflammatory and anti-inflammatory T-cells through secretion of TGFβ, hepatocyte growth factor, PGE2, IDO and nitric oxide.^{183,185} The combination of MSC secreted factors and M2 macrophages can also induce T-regulatory cells that function to suppress overactive adaptive immune response. Taken together, MSCs play an active role in regulating innate and adaptive immunity, thereby calibrating the inflammatory response to disease/injury.

Paracrine Factors of MSCs

There are a variety of paracrine factors present within the MSC secretome including matrix components, enzymes, inhibitors, growth factors, cytokines, chemokines and transcription factors.^{186,187} Accordingly, the MSC secretome contains an array of molecules that have benefits beyond modulating the immune system. For example, MSCs induce anti-apoptotic and pro-survival effects by release of Akt, hypoxia-inducible factors, hepatocyte growth factor, keratinocyte growth factor, insulin-like growth factor 1, and many others.^{186,188} These same growth factors secreted by MSCs can also prevent fibrotic tissue development during repair. Expression of vascular endothelial growth factor (VEGF), fibroblast growth factors, angiopoietins 1/2 and stromal cell-derived factor 1 promote neovascularization and aid in vascular network remodelling within damaged tissues/vasculature.¹⁸⁸ The MSC secretome also demonstrate an ability to activate resident stem/progenitor cell populations to stimulate proliferation and differentiation.

1.13.4 Clinical Potential of MSCs

Despite the mounting preclinical evidence for efficacy of MSCs, there were concerns regarding the safety of administering a live cell therapy to human patients. These theoretical worries included potential malignancy due to the proliferative ability of MSCs, cellular micro-embolism, increased risk of infection given MSC's immunosuppressive effects, and immunogenicity of the cells themselves.¹⁸⁹ The first clinical trial involving MSCs was conducted in 1995 to investigate their feasibility and safety profile.¹⁹⁰ No adverse reactions were observed, and it has since been suggested that MSCs are immune-privileged due to their low expression of major histocompatibility complex proteins.¹⁹¹ A meta-analysis examining the safety of MSCs from clinical trials found no evidence of organ system complications, infections, tumorigenesis or mortality, although MSC infusion may be associated with transient fever.¹⁹² Indeed, the advantageous safety profile of MSCs combined with their multilineage potential, ease of cell

expansion *in vitro* and immunomodulatory abilities have led to over 1000 registered clinical trials for a wide range of indications, including septic shock,¹⁹³ ARDS,¹⁹⁴ ischemic heart disease¹⁹⁵ and most recently, COVID-19 related ARDS (e.g. NCT04400032, NCT04865107). Despite enormous interest, MSCs have only been approved for treating two conditions: (1) graft versus host disease in Canada, Japan and New Zealand, and (2) perianal fistulas in adults with Crohn's disease within the European Union.¹⁹⁶ This lack of success in clinical translation may be attributed to inherent challenges with developing live cell therapies including variabilities in manufacturing, the need for cryopreservation and difficulties achieving adequate cell viability for efficacy. Furthermore, there remains a need for more consistent characterization of MSC therapies, as well as standardized *in vitro* potency assays that can accurately predict the therapeutic efficacy in humans.

1.13.5 MSCs as Therapy for ARDS

The pathophysiology of ARDS involves overwhelming inflammation, alveolar disruption and microvascular injury that is most commonly due to viral/bacterial infection. Given the immunomodulatory, pro-angiogenic, anti-apoptotic and antimicrobial properties of MSCs, research and development of these cells as a biotherapeutic for ARDS has advanced considerably over the past fifteen years.¹⁸¹ One of the first respiratory studies of MSCs exhibited their ability to reduce fibrosis and inflammation in a bleomycin-induced mouse model of lung injury.¹⁹⁷ Since then, a variety of ALI models have discovered MSCs to drastically attenuate pulmonary edema, levels of inflammatory cells/cytokines, vascular permeability and histological lung injury.^{181,198-200} A meta-analysis of small animal models demonstrated a marked reduction to odds of mortality in animals treated with MSCs as compared to control.²⁰¹ This finding remained valid regardless of MSC source, route of administration or method of disease induction. In sheep models of ALI, cell treatment reversed hypoxemia, significantly attenuated lung edema, improved pulmonary vascular

pressures and reduced inflammation with no adverse effects observed.^{202,203} Intrapulmonary infusion of MSCs in *ex vivo* perfused human lungs injured with endotoxin or live bacteria decreased neutrophil accumulation, enhanced macrophage phagocytosis of bacteria, restored alveolar fluid clearance and improved alveolar-endothelial barrier integrity.^{204,205}

Based on the encouraging preclinical outcomes, investigators have begun performing clinical trials involving MSCs for treatment of ARDS. Currently, there are 44 clinical trials registered on ClinicalTrials.gov. Of these studies, 31 are registered specifically for the treatment of COVID-19 associated ARDS, while 13 trials are focused on ARDS from other causes. Five clinical trials have been completed, two of which were early-phase clinical trials that showed multiple infusions of umbilical cord-derived MSCs (90-100 million cells per dose) to be safe in patients with COVID-19 ARDS (NCT04400032 and NCT04355728). Moreover, a recent living systematic review and meta-analysis highlighted the potential efficacy of MSCs to reduce mortality in patients with severe COVID-19.²⁰⁶ A larger clinical trial of MSCs for classical ARDS, the START Study, was recently published in 2019.²⁰⁷ In this randomized phase 2a multicentre trial, allogeneic MSCs were well tolerated by patients with no severe adverse events. However, clinical outcomes including mortality did not improve with MSCs as compared to placebo.²⁰⁷ From post-hoc analysis, one potential reason for the lack of overall efficacy was the widely inconsistent viability of administered MSCs (36-85%). This highlights the difficulties in optimizing methods to produce a clinical grade MSC treatment. Issues pertaining to cell potency, viability, high manufacturing costs and product variability remain at large when attempting to translate live cell therapies. An emerging solution to overcome these barriers includes the idea of isolating and utilizing the MSC secreted factors as an independent therapy.

1.14 Extracellular Vesicles (EVs)

Historically, MSCs were thought to migrate to and engraft at sites of injury, followed by differentiation into functional cells for tissue regeneration. However, a pivotal study in 2005 by Dr. Victor Dzau's group provided evidence that MSC efficacy is facilitated through a novel mechanism involving paracrine action from conditioned media.²⁰⁸ Since then, many studies have confirmed that the therapeutic effects from MSCs do not involve engraftment.²⁰⁹⁻²¹² Rather, MSCs achieve their restorative properties in part by secretion of micro- to nano-sized particles known as extracellular vesicles (EVs).²¹³⁻²¹⁷ EVs are secreted by all cells in the body under physiologic and pathologic states. They carry biologically active cargo encapsulated within their phospholipid bilayer membrane including nucleic acids (microRNAs, mRNAs, mitochondrial DNA), enzymes, growth factors, and lipids. By delivering their cargo to target sites, EVs facilitate intercellular communication that promote homeostasis or disease progression depending on the parent source and packaged content. Thus, EVs represent an opportunity to harness the benefits of MSCs as an “off-the-shelf” cell-free therapy. In turn, MSC-derived EVs (MSC-EVs) may overcome challenges that hamper the successful translation of live cell treatments including limited shelf-life, the need for cryopreservation and batch-to-batch variability in cell viability and/or efficacy.

1.14.1 Biogenesis and Subclasses of EVs

EVs are categorized into three distinct subtypes based on size distribution:²¹⁸ (1) Small EVs (including “exosomes”), ~30–150 nm; (2) medium/large EVs (also known as microvesicles or ectosomes), ~150–1,000 nm; and (3) apoptotic bodies derived from membrane blebbing during programmed cell death, ~1,000–5,000 nm. Small and medium/large EVs are abundantly secreted by MSCs and have received the greatest attention for therapeutic potential (**Figure 1.4**).^{219,220} EVs, whether small or large, can be created through the budding of cellular membrane, enabling for

encapsulation of the cytosolic content of MSCs.²²¹ Many of the current protein markers that are used for EV characterization are surface membrane proteins (e.g. flotillin, caveolin). However, the biogenesis of exosomes has been demonstrated to be quite distinct involving the coupling of endocytosis and exocytosis.²²¹ Within intracellular late endosomes (also known as multivesicular bodies), invagination of the endosomal membrane forms intraluminal vesicles. Once late endosomes fuse at the cell surface, the intraluminal vesicles released into the extracellular environment are recognized as exosomes. Hence, speculated exosomal markers include proteins related to intracellular trafficking and the endosomal pathway (e.g. CD63, CD81, TSG101 and Alix) (**Figure 1.4**).^{216,221} Depending on the EV subtype and their biosynthetic mechanism, the intravesicular bioactive factors can range considerably. Nonetheless, EVs may be an ideal vessel to encapsulate therapeutic products originating from MSCs, protect these products from degradation and aid in their delivery to specific tissue sites.

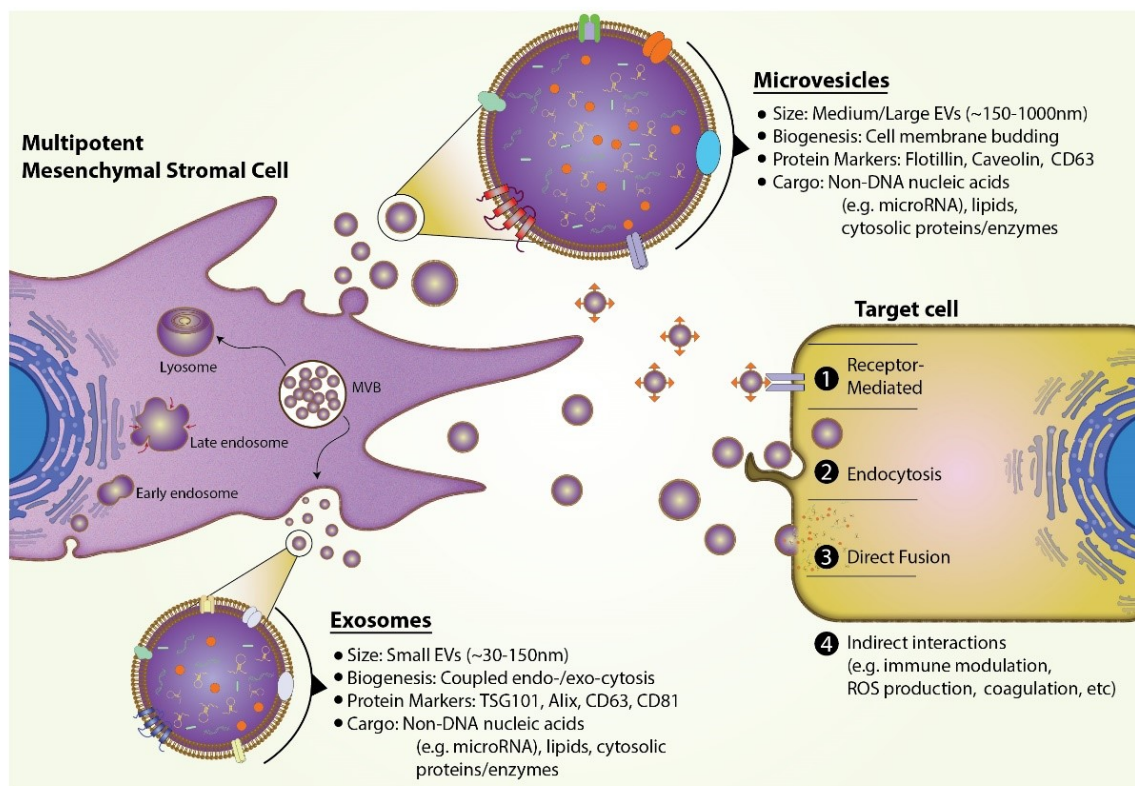


Figure 1.4. MSC-EV subtypes and biogenesis.

Size, biogenesis, protein markers, and vesicular cargo for both exosomes and microvesicles are described. Potential mechanisms by which extracellular vesicles communicate with target cells include receptor-mediated interactions, cellular endocytosis, direct fusion with cellular membrane, and indirect interactions such as immune modulation, reactive oxygen species production, and coagulation. *MVB* – multivesicular body; *TSG101* – tumor susceptibility gene 101; *ROS* – reactive oxygen species. Adapted from Tieu *et al.* (2019).²²²

1.14.2 EV Nomenclature

The first evidence pointing to the existence of EVs dates back to 1946 when platelet-derived particles known as “platelet dust” were shown to be pro-coagulant.^{223,224} It was not until 1981 when the term “exosome” was used in reference to EVs derived from multivesicular bodies found within normal and cancer cell lines.²²⁵ Although the term “exosome” was initially intended to be applied to EVs of endosomal origin, the rise in EV research has found the term being increasingly used to refer to EVs in general, without providing adequate evidence that these are indeed exosomes.²²⁶ Hence, the International Society for Extracellular Vesicles (ISEV) released a recent statement to endorse “extracellular vesicle” as the generic term for particles released by cells.²²⁷ Unless investigators can definitively indicate their EV product is derived from the endosomal pathway, the term “exosome” should be avoided. Instead, EV nomenclature should reflect physical (e.g. size such as small or large EVs), biochemical (e.g. CD63+/CD81+ EVs) or cellular origin (e.g. MSC-EVs, endothelial EVs, etc.) characteristics.

1.14.3 Isolation of EVs

During the *in vitro* culturing of cells, EVs are secreted into the media that can then be collected and stored for future isolation. Importantly, a variety of cell culture parameters can affect EV production including choice in supplementation, timing of collection and culture conditions.²²⁸ Most researchers culture cells with serum supplementation; however, serum is known to contain an abundant amount of undesirable EVs and small particles. To avoid contamination of

conditioned media with serum EVs, one can choose to culture cells under serum-free conditions for 24-48 hours, or supplement media with EV-depleted serum.²²⁸

Other considerations for the preparation of conditioned media prior to EV isolation include cell confluency (recommended 70-90% confluency), normoxic versus hypoxic growth conditions, and preconditioning of cells with molecular/chemical stimuli. For example, culturing MSCs under hypoxia has been thought to enhance the production and efficacy of EVs.²²⁹ Alternatively, researchers have also preconditioned MSCs with pro-inflammatory molecules (e.g. TNF α , IFN γ , LPS) to augment the expression of anti-inflammatory factors within EVs.²³⁰⁻²³² Regardless of how MSCs are cultured, it is critically important to record cell viability at time of conditioned media collection, cell passage number and the duration/concentration of preconditioning stimuli to ensure a consistent EV therapy is produced. After conditioned media is acquired, the storage conditions should be noted (e.g. fresh, refrigerated, frozen at -20°C, frozen at -80°C) and avoiding multiple freeze-thaw cycles is imperative to prevent EV rupture and protein denaturation.^{228,233}

Differential Ultracentrifugation

Differential ultracentrifugation is considered one of the first techniques optimized for EV isolation.²³⁴ It remains the most commonly used approach for EV enrichment due to its ease and accessibility.²³⁵ In short, spin cycles at increasing speeds and g-force can pellet various components within conditioned media based on size and density. Initial steps employ lower centrifugal forces to remove live/dead cells, cellular debris and apoptotic bodies.²³⁴ The supernatant is collected at each step and spun at higher speeds to enrich for specific EV subtypes. For microvesicle isolation, a centrifugal force of around 20,000 x g is commonly utilized. Small EVs including exosomes are pelleted at a force of 100,000 x g.²³⁴

Although ultracentrifugation has advantages as a technically straightforward method with relatively high specificity in EV isolation, there are some disadvantages that warrant consideration. The high centrifugal forces and mechanical stress may result in co-precipitation of EVs with proteins/contaminants and EV rupture.^{228,236-238} Adaptations to the technique including sucrose/iodixanol density gradient or sucrose cushion ultracentrifugation has enabled greater purity in the enrichment of EV populations and reduces the damage sustained by EVs at high spin speeds.^{239,240} Notably, the volume of media that can be processed and quantity of EV yield by ultracentrifugation per session is limited. This severely prohibits the use of ultracentrifugation for clinical translation as patients will require a considerably greater dose of EVs as compared to small animals. Thus, despite the widespread use of ultracentrifugation in preclinical research, optimization of other methodologies is required for effective clinical scaleup.

EV Precipitation Kits

With interest for EV research rising, many companies have manufactured user friendly, inexpensive precipitation kits for rapid particle isolation such as ExoQuick by System Biosciences, Total Exosome Isolation by Invitrogen or exoEasy Maxi Kit by Qiagen. The majority of these kits utilize polyethylene glycol or other water absorbent polymers to precipitate less soluble components such as EVs.²⁴⁰ The advantage of polymeric precipitation is their simplicity and efficiency for high yield isolation of EVs from large volumes of starting biofluid material or conditioned media. However, other non-EV contaminants (e.g. proteins, polymer material, etc.) are extracted in parallel, thereby reducing the purity of the final product.^{239,240} A recent study demonstrated that soluble factors contaminating the EV product may be the dominant component that drives therapeutic benefit.²⁴¹ Hence, efficacy from MSC-EVs isolated by precipitation kits can be incorrectly attributed to EVs rather than the co-precipitated proteins. These unidentified

components and the impure final EV product may limit the ability to obtain regulatory approval for EV therapies isolated by precipitation kits.

Tangential Flow Filtration

Ultrafiltration is a size-based exclusion technique that commonly precedes a primary method for EV isolation. This approach applies dead-end filtration whereby conditioned media is forcibly directed at a semipermeable membrane with a specific pore size.²⁴⁰ As more volume of media is filtered, the membrane becomes saturated with larger solutes resulting in resistance to any further passing of EVs for isolation. Tangential flow filtration (TFF) is a more recent technique for EV enrichment that is increasing in popularity and overcomes issues of ultrafiltration. In TFF, the flow of conditioned media is perpendicular to pores on a hollowfiber membrane. Solution is continuously circulated thereby preventing buildup of molecules around the membrane.²³⁹ Solute and portions of the fluid are permeated through the pores whereas particles larger than the pore size (e.g. EVs) are retained in the TFF system to be concentrated. The remaining EVs can be further purified through a diafiltration process involving the circulation of PBS to remove salts or other media components. This approach has been reported to increase the efficiency for EV isolation upwards of 30-200 fold as compared to ultracentrifugation with added benefits of minimal mechanical stress and protein aggregation.^{242,243} TFF also enables large volumes of conditioned media to be continuously and automatically processed, while retaining expression of EV markers and functional activity in the final product. Taken together, TFF is viewed as a potential scalable technique to produce purified MSC-EVs for clinical application.²⁴⁴

1.14.4 EV Characterization

In 2017, the knowledgebase known as Transparent Reporting And Centralizing Knowledge in EV Research (“EV-TRACK”) was created as an international platform to document and

consolidate the experimental approaches being utilized in EV research.²⁴⁵ Reports from this database suggested high heterogeneity in how researchers were isolating and characterizing EVs. Many articles contained insufficient details, thereby hindering study interpretation or replication. These findings emphasized the need to update the international guidelines for EV experimentation. ISEV published the Minimal Information for Studies of EVs in 2018 (MISEV 2018), which features detailed recommendations to improve transparency and standardization in EV isolation, characterization and study design.²²⁷ Over 380 researchers were surveyed to develop a consensus and synthesize this position statement. The ISEV advises that EV researchers characterize their product by: (1) Quantification, (2) protein composition, and (3) single vesicle analysis. For quantification, total particle number and/or protein amount are frequently used as measures for dosing *in vitro* and *in vivo*. EV particle concentration can be measured using light scattering technologies (e.g. nanoparticle tracking analysis), resistive pulse sensing or flow cytometry for larger EV subtypes. Protein concentrations are typically measured by colorimetric assays including Bradford or bicinchonic acid (BCA) assays. When isolation techniques of low specificity are used (e.g. precipitation kits), assessing the ratio of particle:protein in EVs allows for estimation of purity.²⁴⁶ Often times, lower ratios indicate high co-precipitation of protein contaminants that may result in inconsistent efficacy.

The ISEV guidelines acknowledge a lack of robust evidence to propose universal protein markers corresponding to specific EV subtypes. Rather, MISEV2018 recommends characterizing EVs by evaluating both the positive expression of three general EV markers (e.g. tetraspanins like CD63/CD81, membrane binding proteins including tumor susceptibility gene 101 (TSG101), ALIX, flotillins or caveolins) and negative expression of non-EV proteins (e.g. apolipoproteins, albumin, or proteins related to the endoplasmic reticulum or Golgi apparatus). Lastly, single

vesicle analysis by high resolution visualization and/or non-imaging techniques should be performed. Fluorescence microscopy may be applicable for larger EVs (>200nm), whereas all EVs can be visualized by electron microscopy due to their characteristic lipid bilayer, cup-shaped morphology and immunostaining. Non-imaging single-vesicle modalities that can assess biophysical features (e.g. EV size and concentration) include light scattering technologies by particle displacement, or resistive pulse sensing by electric field displacement.

Another important consideration endorsed by the ISEV is to investigate the function of different EV subtypes before claiming outcomes are associated with only one particular subset.²²⁷ For example, studies that claim therapeutic benefit is due to small EVs or “exosomes” should retain medium/large EVs from the same isolation fraction for side-by-side comparisons of efficacy. If found to be equal, then the rigorous isolation techniques needed to generate an enriched product of small EVs may not be necessary. Additionally, attributing effects of EVs to specific vesicular components should be verified by conducting knock-out or knock-down experiments. This can be achieved by attenuating the expression of target RNAs or proteins within the parent cell prior to EV isolation, or directly altering the composition within EVs. If the original effect is abolished, then one can conclude that the biological factor of interest is indeed crucial to EV activity.

1.14.5 MSC-EVs as a Cell-Free Therapeutic

Although MSC research has dominated the field of regenerative cell therapy, there has been a recent shift in focus to develop MSC-EVs as a novel cell-free nanotherapeutic. Preclinical interventional studies using MSC-EVs are growing at an exponential rate.²⁴⁷ As mentioned, MSC-EVs are thought to deliver protective proteins, microRNAs, mRNAs and lipids to target tissue that regulate immune response, promote tissue repair/regeneration and improve organ function. Several recent systematic reviews and meta-analyses by our group and others highlight the ability of MSC-

EVs to treat a variety of disorders including musculoskeletal diseases,^{248,249} tumor growth,²⁵⁰ wound injury,^{251,252} kidney injury,²⁵³ stroke,²⁵⁴ and cardiac disease.²⁵⁵ In particular, proteomic analysis of MSC-EVs indicate high expression of growth factors and proteins that are pro-angiogenic, immunomodulatory, anti-oxidative and anti-apoptotic.²⁵⁶⁻²⁵⁹

1.14.6 MSC-EVs for Treatment of Respiratory Diseases

Inflammation and microvascular injury are critical to the progression of numerous pulmonary diseases. Hence, MSC-EVs are attractive as a treatment for a range of acute and chronic lung disorders.²⁴⁷ For example, bronchopulmonary dysplasia is a chronic lung disease affecting preterm infants with no effective therapy. It is characterized by restrictions in alveolar and lung vascular development, resulting in significant pulmonary dysfunction. In rodent models, early administration of MSC-EVs markedly reduced inflammation, fibrosis and vascular remodeling while improving lung development.²⁶⁰ A follow-up study that delivered EVs as a rescue therapy found that the regenerative properties of MSC-EVs were sustained despite being given after disease development.²⁶¹ In the adult and elderly population, pulmonary arterial hypertension is a chronic lung vascular disease characterized by endothelial cell apoptosis and excessive proliferation of smooth muscle cells.²⁶² As a result, elevated pulmonary vascular resistance can cause right heart failure. In preclinical models, systemic injection of MSC-EVs was successful at preventing and reversing the development of pulmonary arterial hypertension.^{263,264} Authors attributed these benefits to restoration of mitochondrial health, increased recruitment of immunosuppressive macrophages, and augmented angiogenesis within the lung vasculature.

In preclinical ALI models, MSC-EVs have been shown to as potent as the parent MSCs.²⁶⁵ Several groups found MSC-EVs to markedly improve features of lung injury including histological appearance, inflammatory cell infiltration into lung tissue, and cytokine concentration within the

bronchoalveolar lavage fluid (**Figure 1.5**).^{265,266} Uptake of EVs by alveolar macrophages regulates the *in vivo* inflammatory cascade by polarizing the cells to an anti-inflammatory phenotype. In a recent study by Wang *et al.* (2020), microarray analysis identified microRNA-27a-3p as a critical factor within MSC-EVs for immunomodulation.²⁶⁷ Authors found a loss in MSC-EV efficacy after knocking down microRNA-27a-3p packaging, concluding that microRNA-27a-3p is transferred from MSCs to alveolar macrophages by EVs to drive cellular polarity. This finding adds to a growing understanding of the mechanism by which MSC-EVs exert their benefits during ALI. However, it is pertinent to note that there are a large number of distinct biologically active factors within EVs that may be contributing to their favorable properties in parallel. Other microRNAs shown to play a role in attenuating lung injury include microRNA-21-5p (anti-oxidative effects),²⁶⁸ microRNA-30b-3p (anti-inflammatory and regenerative effects),²⁶⁹ and microRNA-146a (anti-inflammatory and pro-survival effects in a sepsis model) (**Figure 1.5**).²³² Furthermore, the transfer of crucial proteins and mRNAs to target cells have also been implicated including keratinocyte growth factor,²⁷⁰ cyclooxygenase 2,²⁷¹ and angiopoietin-1.²⁷² Aside from macrophage polarization, studies also report that MSC-EVs promote pulmonary angiogenesis in response to microvascular injury,²⁷³ enhance lung epithelial repair to restore barrier function,²⁷⁰ and transfer mitochondria to improve cellular energetics.²⁷⁴

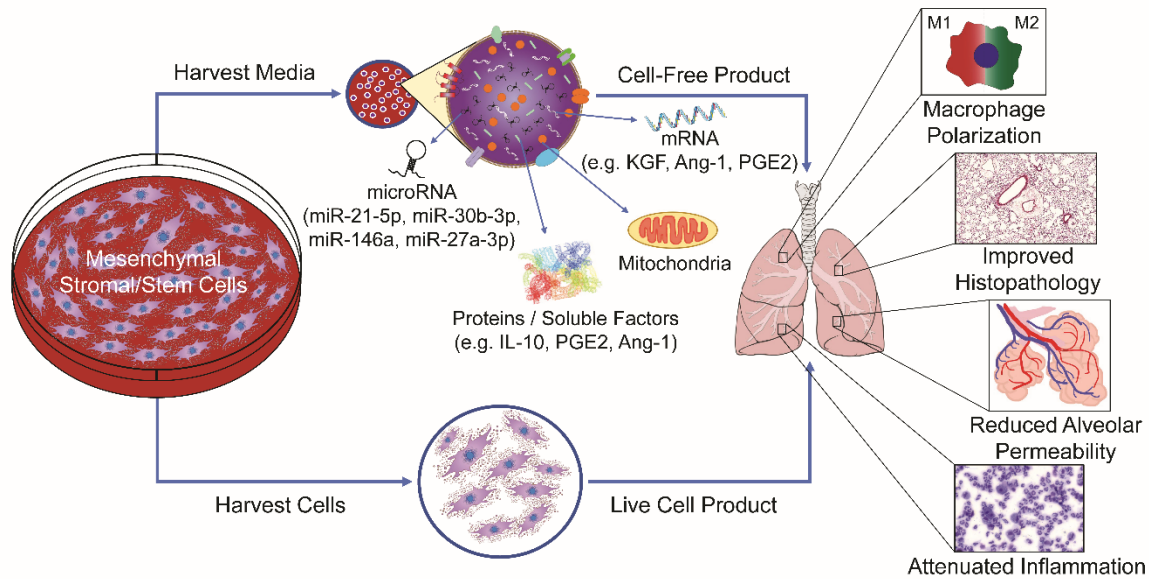


Figure 1.5. MSC-EVs as a therapy for acute lung injury.

Biologically active factors within MSC-EVs that have been shown to mediate beneficial effects include microRNAs, messenger RNAs, proteins, and mitochondrial transfer. In preclinical animal models, MSC-EVs polarize macrophages from the M1 pro-inflammatory to M2 anti-inflammatory phenotype, improve histological lung injury, reduce alveolar-capillary barrier permeability, and attenuate lung inflammation. Ang-1 = angiopoietin-1, IL-10 = interleukin-10, KGF = keratinocyte growth factor, PGE2 = prostaglandin E2. Image adapted from Tieu, Stewart and Lalu (2020).²⁷⁵

In a human *ex vivo* lung perfusion model of ischemia-reperfusion lung injury, MSC-derived microvesicles accelerated pulmonary fluid clearance, restored lung compliance and reduced pulmonary pressures.²⁷⁶ When co-administered with a CD44 neutralizing antibody, all therapeutic effects were suppressed suggesting an important role for CD44 in mediating pulmonary protection by MSC-EVs. In a follow-up study, MSC microvesicles were investigated for their efficacy in treating an *ex vivo* human lung model of severe *E. coli* pneumonia.²⁷⁷ EV treatment attenuated alveolar edema, re-established the endothelial-epithelial barrier and reduced bacterial load. Interestingly, preconditioning of MSCs with an immunostimulant, polyinosinic:polycytidylic acid, produced MSC-EVs with enhanced anti-inflammatory and antibacterial activity.

1.14.7 MSC-EVs: Clinical Potential with Translational Challenges

To date, there are 19 clinical trials of MSC-EV therapy for ARDS registered on ClinicalTrials.gov. Two early phase trials (NCT04544215, NCT04602104) from China focus on the safety of allogeneic MSC “exosomes” for patients presenting with ARDS or bacterial lung infection. Due to the COVID-19 pandemic, an additional six trials are investigating the safety and preliminary efficacy of MSC-EVs for COVID-19 associated ARDS (NCT04276987, NCT04491240, NCT04493242, NCT04798716, NCT04602442 and NCT04657458), two of which have since been completed. One trial with posted results (NCT04491240) indicates that MSC-EV administration is not associated with adverse events. As compared to placebo, no improvements in time to clinical recovery were observed, although larger clinical trials are required for adequate power to determine efficacy. The broad preclinical applicability of MSC-EVs have also led to clinical trials for a diverse number of diseases including diabetes, graft versus host disease, bronchopulmonary dysplasia, stroke and ophthalmic disorders.^{278,279}

Although more investigators are now attempting to translate MSC-EVs from the bench to bedside, these clinical trials may be premature. Like MSC therapy, inconsistencies in EV source and culture conditions as well as non-standardized approaches for isolating and characterizing EVs have hindered interstudy comparisons and progress. There are many unanswered questions within the EV field that need to be addressed to ensure resources are not wasted by attempting clinical trials of poor rigor. For one, there is still no consensus on the optimal isolation technique to efficiently and consistently produce EVs of high purity. Many studies baselessly claim their product as “exosomes” while disregarding the potential efficacy of medium/large EVs. Hence, a greater understanding of whether therapeutic differences exist between EV subtypes is required.

There is currently insufficient data pertaining to the pharmacokinetics of MSC-EVs. Moreover, the biodistribution of EVs is known to be variable and dependent on cell source, route of administration and disease of interest,²⁸⁰ which suggests that pharmacokinetic studies are essential to confirm tissue targeting. Identifying a pragmatic approach for dosing EVs, calculating effective EV dosages and establishing the most efficient route of delivery are important considerations prior to clinical use. Lastly, it is well recognized that the protective capabilities of MSC-EVs can be augmented through preconditioning and genetic engineering. However, a thorough overview of the specific molecular mediators involved in MSC-EV function is necessary for investigators to accurately distinguish the most essential bioactive factor(s) to enhance EV efficacy. It would be incredibly difficult to compare all the different EV techniques and intervention characteristics within one study. Given the extensive number of articles that have now been published on MSC-EVs, meta-research methodology offers one approach to coalesce all the current data available and begin addressing some of these important issues to help advance the EV field.

1.15 Systematic Reviews and Meta-Analyses in Preclinical Research

As with any new field of research, an overwhelming number of narrative reviews have been published to discuss the therapeutic potential of MSC-EVs. Many of these articles focus on pre-specified studies and topics making narrative reviews susceptible to bias as only select information is considered.²⁸¹⁻²⁸³ In clinical research, systematic reviews and meta-analyses are relied upon as methods for rigorous, reproducible, transparent and unbiased evaluations.²⁸⁴ They provide a robust overview on the therapeutic efficacy of new interventions and can help identify points of contention within the field. Importantly, incomplete reporting and inadequate application of study design parameters in preclinical research are recognized as contributors to

irreproducibility over the past decade.²⁸⁵⁻²⁸⁹ In conducting systematic reviews, an assessment on risk of bias is often completed, enabling us to determine aspects of experimental design that require greater attention to ensure threats to validity are minimized.

As our understanding of EV biology advances, establishing standardized, reproducible techniques for studying and defining EVs is critically important. However, there is considerable heterogeneity in the scientific approach, experimental protocols and outcomes of EV studies. By applying systematic review methodology, an overview of the commonly applied techniques can help guide design of robust preclinical studies. Meta-analyses of outcomes in respiratory illnesses may determine a more accurate estimate for the effectiveness of MSC-EVs. Lastly, a comprehensive assessment of the current methodology and preclinical findings will identify crucial knowledge gaps to help direct future research and hopefully accelerate the clinical translation of this novel cell-free therapy.

1.16 Summary

Many of the therapeutic benefits observed from MSCs are thought to be mediated by the secretion of EVs at the site of injury. Research into MSC-derived EVs as a cell-free therapy is growing at a rapid rate due to their potential for greater consistency and favorable processing profile. By using a meta-research approach, a comprehensive synthesis of current experimental techniques, mechanisms of action and general outcomes can improve our insight on the function of MSC-EVs. Determining whether MSC-EVs can be used as a viable therapy for lung injury and understanding its biodistribution during ALI will generate a more complete picture on how and when EVs should be administered for optimal efficacy. With the SARS-CoV2 pandemic causing worldwide disruption to scientific progress including my own research, a distinct objective was

added to my thesis to help combat COVID-19. My experience in preclinical modeling of ALI helped design experiments to establish a novel, more widely accessible animal model of COVID-19 associated ARDS. In turn, I hope my findings can facilitate a better understanding of COVID-19 pathogenesis and enable widespread investigation into new interventions.

1.17 Objectives and Hypotheses

1.17.1 COVID-19 Impact on Research Objectives

Given lab and campus closures during the COVID-19 pandemic that caused considerable delays to research, my thesis was updated to include dry lab projects involving systematic reviews and meta-analyses with a preclinical focus on MSC-EVs. These changes allowed me to maintain productivity during the pandemic as I was able to publish three manuscripts (Objectives 1 and 2) with an additional two manuscripts currently in preparation (Objectives 3 and 4). Conducting these systematic reviews have helped me identify a considerable knowledge gap in biodistribution data within the field of MSC-EVs. Hence, Objective 3 focuses on determining the biodistribution and kinetics of MSC-EVs during acute lung injury. Lastly, we have also added an Objective 4 which involves a collaboration with Dr. Carolina Ilkow (Ottawa Hospital Research Institute) to develop a novel mouse model of COVID-19 associated ARDS.

1.17.2 Objective 1 – An Analysis of MSC-EVs for Preclinical Use (Chapters 2 and 3)

Objective 1 is an exploratory analysis of MSC-EVs in preclinical animal studies. Using a systematic review approach, I aimed to: (1) create a comprehensive evidence map of methodology applied for *in vivo* studies evaluating MSC-EVs as an intervention, (2) determine the molecular factors and cellular pathways critical for MSC-EV efficacy, and (3) summarize preclinical outcomes as a result of MSC-EV administration.

Rationale: This study updated and expanded on what was previously published by Akyruekli in 2015.²¹³ At that time, only 17 studies were included, but the findings highlighted a potential for EVs as a novel therapy. Since then, there has been an enormous increase in research interest to investigate the therapeutic properties and biological mediators of MSC-EVs.

1.17.3 Objective 2 – Systematic Review and Meta-Analysis of MSC-EVs for Treatment of Respiratory Diseases (Chapter 4)

In Objective 2, it was hypothesized that MSC-EVs will reduce injury, attenuate inflammation and improve organ function for preclinical ALI. Hence, I aimed to: (1) conduct a systematic review and meta-analysis of MSC-EVs as therapy for lung conditions, (2) assess the risk of bias in MSC-EV preclinical studies, and (3) evaluate the quality of reporting in accordance with the National Institute of Health (NIH) guidelines. I also performed exploratory subgroup analyses to determine the effect of different EV characteristics on therapeutic efficacy.

Rationale: Given the widespread number of approaches that can be used to study EVs, there is no current consensus on which tissue source of MSCs, isolation techniques or EV subtype result in greatest efficacy. Hence, a meta-analysis was used to quantitatively determine which respiratory conditions, acute or chronic, can benefit from MSC-EV administration. Moreover, subgroup analyses helped elucidate the most efficacious intervention characteristics.

1.17.4 Objective 3 – Biodistribution and Optimal Timing of Administration of MSC-EVs During ARDS (Chapter 5)

In Objective 3, I aimed to: (1) characterize a mouse model of ALI, (2) determine the optimal time to administer MSC-EVs for lung distribution, and (3) evaluate the kinetics and organ-specific uptake of EVs during ALI. It was hypothesized that lung distribution of MSC-EVs will be greatest during heightened lung permeability and inflammation.

Rationale: There is increasing interest in the use of EVs as therapeutic agents,^{290,291} but little is known about the biodistribution of MSC-EVs especially during ALI. It is believed that EVs commonly accumulate to the liver and spleen for clearance, with less distribution to the lungs and other organs in baseline (non-diseased) conditions. However, under a state of endothelial permeability and inflammation, MSC-EVs may home to sites of injury and extravasate into tissues.²⁹²

1.17.5 Objective 4 – Developing a Novel, More Accessible Model of COVID-19 (Chapter 6)

In Objective 4, it was hypothesized that a SARS-CoV2 pseudotyped virus will induce lung injury in mice similar to COVID-19 ARDS. The goals were to: (1) determine the optimal dose of pseudovirus required to induce lung injury, (2) conduct a time course study to determine temporal changes to animal weight, temperature and mortality, and (3) assess outcomes of preclinical ALI including histological injury and inflammatory response.

Rationale: A Containment Level 3 facility is required to study SARS-CoV2. Unfortunately, these facilities are only available at a limited number of institutes around the world. Hence, by creating a “pseudovirus” of SARS-CoV2 and establishing a novel mouse model of COVID-19 ARDS, we can provide a more accessible method for researchers around the world to investigate the pathophysiology of COVID-19 and evaluate new potential therapies.

**Chapter 2 – Methods and Efficacy of Extracellular Vesicles Derived
from Mesenchymal Stromal Cells in Animal Models of Disease: A
Preclinical Systematic Review Protocol**

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Citation:

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Author Contributions:

AT: Created the systematic review protocol including the research question and methodological approach. Wrote the manuscript and generated the figures/tables.

MML and DSA: Principal investigators of project. Contributed to writing of manuscript, creation of protocol, and finalizing research question and methodological approach.

MS, DAF, JM, DB and DJS: Assisted in developing systematic review protocol and review of manuscript.

RS: Created search strategy of systematic review.

2.1 Abstract

Background: Over the past decade, mesenchymal stromal cells have been increasingly investigated for their therapeutic potential in several different illnesses. However, cell therapy can be limited by potentially serious adverse events including cell embolus formation and tumorigenesis. Importantly, the protective effects of mesenchymal stromal cells are largely mediated by paracrine mechanisms including release of extracellular vesicles. This systematic review intends to synthesize the current knowledge of mesenchymal stromal cell-derived extracellular vesicles as a therapeutic option for preclinical models of disease, inflammation, or injury.

Methods: A systematic literature search of MEDLINE, Embase, and BIOSIS databases was conducted. Interventional preclinical *in vivo* studies using extracellular vesicles derived from any tissue source of mesenchymal stromal cells will be included. Studies will be screened by abstract, and full-text by two independent reviewers. Eligible studies will undergo data extraction with subcategorization into domains based on disease. Methods utilized for extracellular vesicle characterization and isolation will be collected, as well as information on interventional traits, such as tissue source of mesenchymal stromal cells (allogeneic or xenogeneic administration), dosage regimen, and vesicle modifications. Reported outcomes will be collected to determine which research disciplines may be impacted most from treatment with mesenchymal stromal cell-derived extracellular vesicles.

Discussion: This systematic review will summarize preclinical studies investigating the therapeutic efficacy of both small and large extracellular vesicles derived by mesenchymal stromal cells. Extracellular vesicles represent a possibility to harness the benefits of mesenchymal stromal cells with added benefits of reduced manufacturing costs and an improved safety profile. Hence,

there has been an exponential increase in interest for developing this cell-free therapy with hundreds of preclinical studies published to date. However, a vast amount of heterogeneity between groups relate to methods of extracellular vesicle isolation, characterization, and study design. This review will capture this heterogeneity and identify the most commonly used and optimal approaches to evaluate mesenchymal stromal cell-derived extracellular vesicle treatment. A meta-analysis of outcomes within each disease domain will help elucidate which fields of research demonstrate promise for developing extracellular vesicles as a novel cell-free therapy. Summarizing this robust information on microvesicles and exosomes as an intervention can provide guidance for designing preclinical studies with hopes of future clinical translation.

Keywords: Mesenchymal stromal cells, mesenchymal stem cells, exosomes, microvesicles, extracellular vesicles, systematic review protocol, preclinical

2.2 Background

Mesenchymal stromal cells represent a subset of adult stem-like cells which have been extensively investigated for therapeutic potential in a variety of disease states. Mesenchymal stromal cells have been isolated from numerous tissue sources including: adipose tissue, umbilical cord, and synovial membrane,¹⁶⁴ and they can be derived from induced pluripotent stem cells.¹⁶⁵ Their multilineage differentiation potential, *in vitro* expansive capacity, and ability for immunomodulation has led to nearly 500 clinical trials involving septic shock,¹⁹³ acute respiratory distress syndrome,²⁹³ and ischemic heart disease.^{294,295} In particular, mesenchymal stromal cells have received approval as a therapy to treat graft versus host disease in New Zealand, Canada and Japan.^{296,297} Cell therapies may be limited by potentially serious adverse events of cell embolus and tumour formation.²⁹⁸ Thus, there is immense interest in harnessing the therapeutic effects of mesenchymal stromal cells in ways that avoid potential harms.

Mesenchymal stromal cells' effects are largely mediated by paracrine mechanisms, and so mesenchymal stromal cell-derived extracellular vesicles may represent a safer cell-free alternative to whole cell injections. Extracellular vesicles contain biologically active factors and signaling molecules to facilitate intercellular communication. With opportunities for diminished manufacturing and storage costs for extracellular vesicles, they can also be produced as a more readily available "off-the-shelf" product, as extracellular vesicles are more stable than whole cells when cryogenically frozen. Research into the development of cell-free therapies have focused primarily on two types of extracellular vesicles, medium-large vesicles known as microvesicles, and small extracellular vesicles, which include exosomes.

Microvesicles and exosomes are subsets of extracellular vesicles that range from 150-1000 and 30-150 nm in diameter, respectively.²⁹⁹ Both microvesicles and exosomes are prolifically

produced by mesenchymal stromal cells and are believed to carry bioactive cargo, such as microRNA, transcription factors, growth factors, and other regulatory proteins that help mediate their potentially efficacious properties.^{300,301} Extracellular vesicles may serve as ideal vesicles for encapsulating therapeutic products, protect these products from degradation, and aid their delivery to specific target tissues. The opportunity to introduce or manipulate the expression of contents inside extracellular vesicles (e.g. through overexpression of protective microRNAs) give rise to an intriguing new domain of regenerative medicine.

The systematic review stemming from this protocol will synthesize current evidence on mesenchymal stromal cell-derived microvesicles and exosomes as therapeutic options in preclinical models of disease, inflammation, or injury. A scoping review previously published by our group systematically reviewed preclinical data on extracellular vesicles up to 2013 and found that sixteen of seventeen preclinical studies demonstrated therapeutic benefits associated with administration of mesenchymal stromal cell-derived extracellular vesicles.²¹³ Subsequently, the terminology surrounding extracellular vesicle research has matured and interest in studying microvesicles and exosomes has increased exponentially, necessitating a re-evaluation of this body of evidence.

2.3 Research Objectives

We will review *in vivo* preclinical studies evaluating mesenchymal stromal cell-derived extracellular vesicles as an intervention for any animal model of disease or injury. Our primary objective will assess the methodology being utilized in these animal studies including: (1) interventional characteristics, (2) methods of extracellular vesicle enrichment and characterization, and (3) experimental design. Given the expected heterogeneity of methods used (e.g. cell sources,

isolation techniques, etc.) and outcomes assessed across various disease domains, a synthesis of these issues is needed to provide context for any subsequent meta-analysis. For our secondary objective, studies will be categorized by disease domains with subsequent meta-analyses of study outcomes to determine the therapeutic efficacy of mesenchymal stromal cell-derived extracellular vesicles in different conditions. Results of this analysis will help guide future preclinical studies of extracellular vesicles and inform whether possible human participant studies are feasible.

2.4 Methods and Design

2.4.1 Review Team

The team members for this systematic review encompass researchers across different scientific disciplines. The team has expertise in systematic reviews (DAF, MML, JM, DSA), basic science studies investigating novel cell therapies (AT, MML, DJS, DSA), and translational cell therapy trials (DJS, DAF).^{295,302,303} As well, DB participated in development of the recently published Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018) position statement and provides considerable experience in this growing field of extracellular vesicle research.²²⁷ The student leads for this project (AT and MS) have experience in both basic science and clinical epidemiology and will be the two primary reviewers for article selection and data extraction.

2.4.2 Protocol

This systematic review protocol is reported in accordance with the Preferred Reporting Items for Systematic reviews and Meta-Analysis Protocol (PRISMA-P) reporting guidelines (Supplemental File A1).³⁰⁴ A summary of the protocol has been registered at the International Prospective Registry of Systematic Reviews (PROSPERO CRD42019123918).

2.4.3 Eligibility Criteria

Population: Inclusion - *In vivo* preclinical animal models of disease, organ injury, tumour growth, or altered immune response. Exclusion - *Ex vivo*, *in vitro*, and invertebrate animal models will be excluded.

Intervention: Inclusion - Mesenchymal stromal cell-derived extracellular vesicles including microvesicles (150nm to 1µm) and exosomes (30-150 nm). Extracellular vesicles can be derived from xenogeneic, syngeneic, or allogeneic mesenchymal stromal cells from any tissue source. All routes of intervention administration will be considered, including direct tissue, intravenous and intra-arterial injections. Experiments involving pre-treatment of mesenchymal stromal cells, co-treatment and/or genetic manipulation (e.g. over- or under-expression of genes) before extracellular vesicle isolation will be classified as “modifications” for subgroup analysis. Exclusion - Studies where extracellular vesicles are not directly administered to animals as a therapy (e.g. used as a pre-treatment for other interventions) or investigated for their biodistribution will be excluded.

Comparators: Studies with any comparator (e.g. vehicle control, placebo, mesenchymal stromal cells, fibroblast microvesicles) will be considered.

Outcomes: Inclusion – Studies that provide information regarding intervention characteristics, methodologic approaches and experimental design parameters utilized in studies will be included. For the purposes of a potential quantitative synthesis and meta-analysis, we will extract each study’s primary outcome. If a primary outcome has not been outlined in the study, the article’s abstract will be reviewed, and the most prominent outcome will be used for data extraction.

Study Design: Inclusion – Interventional *in vivo* studies with a comparator-control will be included. Studies may be randomized, pseudo-randomized or non-randomized. Exclusion – Unpublished grey literature, abstracts, review articles, editorials, and letters will be excluded. Single arm interventional studies will also be excluded.

2.4.4 Data Sources

We will search Ovid MEDLINE®, Ovid MEDLINE® In-Process & Other Non-Indexed Citations, Embase Classic + Embase, and BIOSIS from inception to present day. In addition, hand searches of the bibliographies of included articles and relevant reviews will be performed.

2.4.5 Search Strategy

The search strategies (Supplemental File A2) to be used for this review will be generated by a Health Sciences Librarian (RS) with experience designing systematic literature searches. A second information specialist with no association to the project will also review the strategy using Peer Review of Electronic Search Strategies (PRESS) before the final search procedure is executed.^{305,306} The search strategy will utilize both calculated vocabulary and MeSH terms (e.g. mesenchymal stem cells, mesenchymal stromal cells, extracellular vesicles, exosomes, microvesicles), acronyms (e.g. MSCs, microvesicles, extracellular vesicles), and MeSH terms with alterations as required per database. Our previous search strategy published in 2015 was updated to reflect evolving terminology in the extracellular vesicle field.²¹³ In addition, preclinical filters will also be applied to improve search efficiency.³⁰⁷⁻³⁰⁹ No search restrictions will be created for language or publication date.

2.4.6 Study Records

Data Management: Search results will be uploaded to Distiller Systematic Review Software (DistillerSR, Evidence Partners, Ottawa, Canada). DistillerSR is an online software that allows for improved transparency, reproducibility and accessibility for literature review.

Selection Process: Two reviewers (AT, MS) will independently screen titles and abstracts from search results using the predefined inclusion criteria outlined above. A calibration test involving a set of 10 studies will be performed (AT, MS) to refine the screening question and ensure high inter-rated correlation ($kappa > 0.8$), prior to formally commencing the screening process. For all titles that appear to meet the inclusion criteria or where there is any uncertainty, we will obtain the full text article. Two reviewers (AT, MS) will assess the eligibility of full-text articles. A calibration test involving a set of 10 studies will be performed (AT, MS) to ensure articles of high inter-rater correlation. After every calibration test, the entire review team will be consulted to resolve any issues concerning full-text inclusion. After refining the full-text screening criteria, a formal screening process (AT, MS) will be commenced. Any disagreement will be resolved through discussion with a third-party member (MML, DSA). Reasons for study exclusion at this level will be recorded. There will be no restrictions on language. Articles of non-English text will be translated by native speakers at the Ottawa Methods Centre.

Data Collection Process: A standardized data extraction form will be designed by the review team and uploaded onto DistillerSR. Information will be extracted independently and in duplicate from each eligible study (AT, MS). Calibration exercises of 20 studies will be conducted, and the review team will be consulted to refine the data extraction form. After commencing formal data abstraction, any disagreements will be first discussed between two independent reviewers (AT, MS). If no resolution can be made, a third-party team member will be consulted (MML).

Data Items: Broad categories of data items include: methods of vesicle separation and characterization (Table 2.1), intervention details (e.g. tissue type of mesenchymal stromal cell, dose, route of administration, and timing of administration) (Table 2.2), study characteristics (e.g. study design, population of species, model of disease) (Table 2.3), and preclinical outcomes.

2.4.7 Risk of Bias Assessment

For each included study, risk of bias assessments will be carried out by two independent reviewers (AT, MS) using the Systematic Review Centre for Laboratory animal Experimentation (SYRCLE) risk of bias tool.³¹⁰ The SYRCLE tool was adapted from the Cochrane Risk of Bias Tool to assess the methodologic quality using criteria specific to animal studies. Items in this tool include assessments for selection bias (sequence generation, baseline characteristics, allocation concealment), performance bias (random housing, blinding), detection bias (random and blinded outcome assessment), attrition bias (completeness of outcome data), and reporting bias. Each parameter of bias for each included study will be scored as having a low, high, or unclear risk of bias. Any disagreements between the two reviewers will be resolved through discussion with a third-party member (MML).

2.4.8 Data Analysis

Before a meta-analysis will be deemed appropriate to execute, the heterogeneity of included studies will be assessed. A senior author (MML, DA, DB) will review the study populations and study characteristics to judge presence of methodologic heterogeneity. If sufficient homogeneity across studies exists, a meta-analysis will be considered appropriate to carry out. Continuous endpoints will be pooled using standardized mean differences with an inverse variance random effects method.^{311,312} Dichotomous outcomes (e.g. mortality) from each study will be pooled and described as risk ratios with 95% confidence intervals using the

DerSimonian and Laird random-effects method (Comprehensive Meta-Analysis v3.1).³¹³ Statistical heterogeneity of effect sizes will be assessed using the Cochrane I^2 statistic test.³¹⁴ Thresholds for interpretation of I^2 are as follows: 0–40% (low heterogeneity), 30–60% (moderate heterogeneity), 50–90% (substantial heterogeneity), and 75–100% (considerable heterogeneity).³¹⁴ If there is considerable heterogeneity (75–100%), sources of heterogeneity will be explored through subgroup and sensitivity analyses.

2.4.9 Subgroup Analyses

Planned subgroup analyses includes intervention characteristics, such as tissue source of mesenchymal stromal cells (e.g. bone marrow, umbilical cord, adipose), size of extracellular vesicles (e.g. small extracellular vesicles from 30-150nm, or medium/large extracellular vesicles from 150-1000nm), method of extracellular vesicle isolation (e.g. ultracentrifugation, tangential flow filtration, size exclusion chromatography), and methods of vesicle characterization (e.g. Western Blot, electron microscopy, flow cytometry). Additionally, studies will be sub-grouped by disease domain for preclinical outcome assessment.

2.4.10 Meta-Biases Assessment

Evaluation for publication bias and selective reporting bias will be conducted using a funnel plot, generated by Comprehensive Meta-Analyst, and Egger's regression test.^{315,316}

2.4.11 Knowledge Dissemination

Understanding that there has been a markedly heightened interest to evaluate the therapeutic potential of mesenchymal stromal cell-derived extracellular vesicles, this review will first assess the feasibility of conducting a meta-analysis based on the final number of included preclinical studies. If a feasible number of articles are included, our team will summarize the methodology data and conduct a meta-analysis of outcomes. If the included number of articles is

too large to feasibly perform multiple analyses in a single publication, a systematic review of the overall methodology being used for *in vivo* extracellular vesicle studies will be first published. Subsequently, analyses for each disease domain with more details (e.g. risk of bias and outcome measures) will be published as separate manuscripts in peer-reviewed journals. Finally, findings will be disseminated through international conferences with basic and translational scientists to help guide preclinical and translational study design of extracellular vesicle therapy.

2.4.12 Amendments

If any amendments to this protocol are necessary, the date and specific changes to the protocol will be documented on PROSPERO with rationales as to why the alterations were required.

2.5 Discussion

Our systematic review intends to identify and summarize preclinical studies investigating the therapeutic efficacy of both small and large extracellular vesicles derived from mesenchymal stromal cells. This work updates our previous study that captured studies through 2013²¹³. Although only 17 studies were included in the previous systematic review, a therapeutic potential for this novel cell-free therapy was evident for extracellular vesicles in multiple states of disease and injury. Since then, many more research groups around the world have begun to investigate extracellular vesicles, and they are well recognized to carry biologically active factors to mediate the therapeutic effects of mesenchymal stromal cells. Extracellular vesicles represent a novel investigative direction within regenerative medicine, especially due to a possibility to manipulate packaged products (growth factors and microRNA).

We anticipate that this comprehensive synthesis and meta-analysis of preclinical studies assessing the beneficial or adverse effects of mesenchymal stromal cell-derived extracellular vesicles will generate valuable data to help guide future preclinical study design. If proven favorable, the robust information on microvesicles and exosomes can hopefully determine whether their therapeutic efficacy is clinically significant. More importantly, these findings can help provide considerations of future translational human participant studies.

2.6 Supplemental Files

Supplemental File A1: Preferred Reporting Items for Systematic review and Meta-Analysis Protocols (PRISMA-P) 2015 Checklist.

Supplemental File A2: Search strategy used for systematic literature search.

2.7 Declarations

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Authors' Contributions

AT, MS, DSA, and MML conceived the study design. AT, DSA, and MML were responsible for the initial drafting of the manuscript. AT and MS formulated the data extraction questions and tables. DJS, DAF, JM, and DB provided important revisions for the protocol

development as well as data extraction questions. MML is the guarantor of this review. All authors were involved in manuscript revisions before final approval.

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Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Availability of Data and Material

Not applicable.

Competing Interests

DJS is President and CEO of Northern Therapeutics (Montreal, QC, Canada). The remaining authors have no competing interests to declare.

Table 2.1. Isolation and characterization of mesenchymal stromal cell extracellular vesicles.

Question of Interest	Example Answers
Terminology Used	Extracellular Vesicles Exosomes Microvesicles Small EVs Large EVs Microparticles
Size of EVs	Small EVs (30-150nm) Large EVs (150-1000nm) Both Used Together Both Used Separately
Method of EV Enrichment	Ultracentrifugation Isolation Kit Tangential Flow Filtration
Method of EV Characterization	Nanoparticle tracking analysis (size) Dynamic light scattering (size) Tunable Resistive Pulse Sensing (size)
	Western Blot (protein content) Flow Cytometry (protein content) ELISA (protein content) Proteomics (protein content)
	Electron Microscopy (morphology) Bradford/BCA Assay
Protein Markers Used	CD63 (seen in many EVs) CD9 (seen in many EVs) CD81 (small EV) TSG101 (small EV) Other (specify)
Negative Protein Markers	Calnexin Cytochrome C

Abbreviations: EV – Extracellular vesicle, ELISA – Enzyme-linked immunosorbent assay, BCA Assay – bicinchoninic acid assay, TSG101 – Tumor susceptibility gene 101

Table 2.2. Interventional traits and dosage regimen of the extracellular vesicle therapy.

Question of Interest	Example Answers
Storage of EVs	Frozen at -80°C Fresh / Fridge Not Described
Tissue Source of MSCs	Bone Marrow Adipose Umbilical Cord/Wharton's Jelly
Animal Source of MSCs	Human Source Mouse Source Rat Source
EV Immunocompatibility	Xenogeneic Allogeneic Autologous Not Described
Modification of EVs	Unmodified Modified MSCs Before EV Isolation Modified EVs Directly
Route of Administration	Intravenous Direct Tissue Injection Subcutaneous Intra-arterial
EV Dosage Units	EV Protein Amount EV Particle Number EV Dose Based on MSC Cell Count
Timing of First EV Dose	Post-injury induction (treatment) Pre-injury induction (prevention)
Number of Doses Used	Single Dose of EVs Multiple Doses of EVs

Abbreviations: EV – Extracellular vesicle, MSC – Mesenchymal stromal cells

Table 2.3. Disease domains, preclinical models, and randomization.

Question of Interest:	Answers:
What was the Disease or Condition of Interest?	Brain (e.g. stroke, traumatic brain injury) Kidney (e.g. acute kidney injury, chronic kidney disease) Lung (e.g. acute lung injury, pulmonary hypertension) Bone/Joint (e.g. osteoarthritis) Liver (e.g. hepatotoxicity, chronic liver disease) Cardiac (e.g. myocardial infarction) Cancer
Animal Model	Mouse Rat Pig
Were Animals Randomized?	Yes No

Chapter 3 – An Analysis of Mesenchymal Stromal Cell-Derived Extracellular Vesicles for Preclinical Use

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Author Contributions:

AT: Created the systematic review protocol, conducted the literature search, assessed study eligibility and completed data extraction. Contributed to data analysis, wrote the manuscript, generated the tables/figures and addressed reviewer comments/revisions.

MML and DSA: Principal investigators of project. Contributed to writing of manuscript, creation of protocol, and finalizing methodological approach. Assisted in addressing reviewer comments/revisions and generating tables/figures.

MS and CG: Assisted in conducting literature search, assessing study eligibility and completing data extraction. Reviewed manuscript for final submission.

DAF and JM: Contributed to data analysis and review of manuscript.

DB and DJS: Assisted in developing systematic review protocol and review of manuscript.

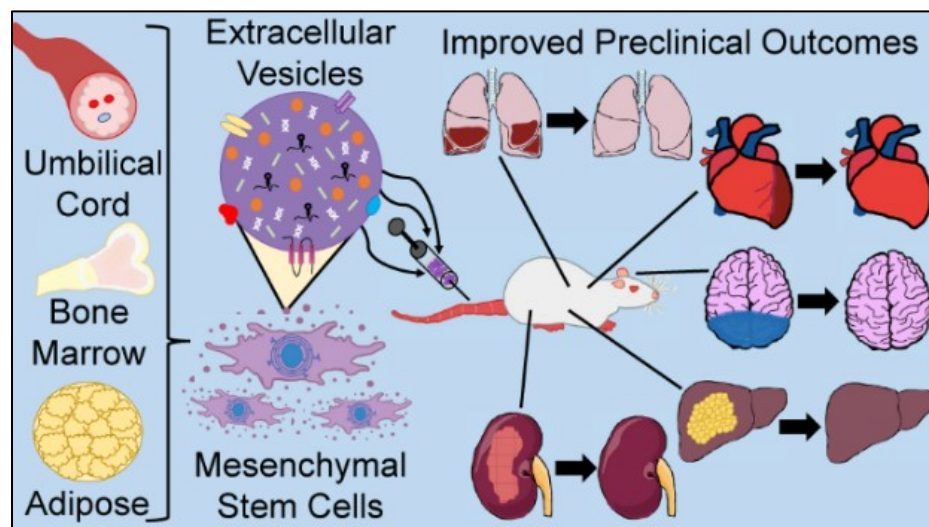
3.1 Abstract

Mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) can reduce inflammation, promote healing and improve organ function thereby providing a potential ‘cell-free’ therapy. Prior to clinical translation, it is critical to synthesize existing evidence on preclinical methods and efficacy. To address these issues, we used gold standard systematic review methodology to consolidate information from all published animal studies investigating MSC-EVs as an intervention. Systematic search of MEDLINE and Embase identified 206 studies. Data was extracted in duplicate for methodology, experimental design, interventional traits, modifications and outcomes. MSC-EVs were used to treat a variety of diseases and demonstrated benefits in 97% of studies. Adverse effects were reported in only three studies; two demonstrating tumor growth. A quarter of articles modified EVs to enhance efficacy with 72% leading to markedly improved outcomes as compared to unmodified EVs. However, several key methodological concerns were evident. Only 60% of studies used nomenclature consistent with the size definitions of EVs. Ultracentrifugation (70%) and isolation kits (23%) were the most common isolation techniques despite differences in yield and purity. EVs were inconsistently dosed by protein (68%) or particle concentration (16%). Two-thirds of studies administered xenogeneic EVs suggesting immunocompatibility. Less than 25% of studies assessed EV biodistribution. Approaches for determining size, protein markers and morphology was highly heterogeneous with only 12 and 4 studies meeting the MISEV 2014 and 2018 recommendations, respectively. Knowledge gaps identified from this systematic review highlight important opportunities to improve preclinical design and methodology in the rapidly growing field of EV therapeutics.

Keywords

mesenchymal stem cells, extracellular vesicles, exosomes, microvesicles, preclinical, systematic review

Graphical Abstract:



3.2 Introduction

Mesenchymal stromal cells (MSCs) are a subset of adult-like stem cells that can be derived from a variety of tissue sources including bone marrow, adipose tissue and umbilical cord, or even differentiated from pluripotent stem cell populations.^{165,317} MSCs have been widely studied in animal models and clinically due to their immunomodulatory capabilities. Despite enormous interest, MSCs have only been approved for treating two conditions in a limited number of countries: (1) graft *versus* host disease in Canada, Japan, and New Zealand, and (2) perianal fistulas in adults with Crohn's disease within the European Union.¹⁹⁶ This may reflect the technical challenges faced in developing a viable and potent cell product compounded by heterogeneity in study design that has complicated regulatory approval.³¹⁸ For example, post-hoc analysis from a recent phase IIa clinical trial of MSCs for treatment of acute respiratory distress syndrome demonstrated a considerable range in cell viability from 36 to 85 %.²⁰⁷ This highlights the pertinent difficulties in optimizing methods to produce a clinical grade cell therapy.

Interestingly, the beneficial effects of MSCs do not require cell engraftment.²⁰⁹⁻²¹² Instead, MSCs achieve their restorative properties through paracrine mechanisms including the secretion of extracellular vesicles (EVs).^{213-215,217} EVs are membrane-enclosed submicron particles released by all cell types into the extracellular environment.²¹⁶ Identifying, amplifying and concentrating the effectors of MSC therapy may serve as a feasible route for therapeutic development. By packaging an assortment of biologically active factors (lipids, microRNAs, growth factors, regulatory proteins), EVs have been implicated as key modulators of intercellular communication for physiological and pathological processes. Accordingly, they represent a possibility to harness the therapeutic benefits of MSCs as a potentially cell-free cell therapy. EVs may be formulated as an “off-the-shelf” intervention to circumvent many of the challenges inherent to whole cell

injections including cell viability, high manufacturing/storage costs, and batch-to-batch product variability.

The International Society of Extracellular Vesicles (ISEV) has recently published guidelines on the Minimal Information for Studies of Extracellular Vesicles (MISEV).²²⁷ The authors encouraged the use of generic terminology including categorization of EVs by physical characteristics. Research into the use of MSC-derived EVs as a therapy have primarily focused on two major subclasses: (1) medium-large EVs (more commonly referred to as “microvesicles”) and (2) small EVs (which include “exosomes”). It is known that MSCs prolifically produce both subtypes of EVs.^{219,220} The major distinguishing feature between medium/large and small EVs is their approximate size distribution ranging from ~150-1000nm and ~30-150nm, respectively.²²⁷

EVs, whether small or large, can be created through the budding of cellular membrane, enabling for encapsulation of cytosolic content of MSCs. Hence, many of the current markers suggested for confirming their isolation are surface membrane proteins such as flotillin and caveolin. Conversely, exosomes are a subclass of small EVs generated by coupling endocytosis and exocytosis through the formation of multivesicular bodies, which corresponds to markers related to intracellular trafficking and the endosomal pathway (*e.g.* CD63, CD81, TSG101 and Alix). In addition to terminology, size parameters and protein markers, the MISEV 2018 guidelines highlight many other salient considerations necessary for drawing conclusions on the function of EVs.²²⁷ This updated ISEV statement reflects an improved understanding in EV biology, which has resulted in a consensus to promote meaningful changes to nomenclature and experimental approach. Despite these recommendations, many aspects for how EVs are isolated, characterized and administered remain highly variable. Like all fields of preclinical research, more transparent reporting of EV experimentation will ensure study validity, accurate findings, and reproducibility.

The EV-TRACK Platform was developed as an opportunity to centralize data with a goal of standardizing EV research.²⁴⁵

Given the rapid growth of publications reporting on MSC-derived EVs, a synthesis of published studies is needed to gain an objective understanding of which approaches appear effective, and to identify barriers that hinder progression into human studies. We performed a systematic review to analyze studies administering MSC-derived EVs as an intervention for *in vivo* animal models of disease. Unlike narrative reviews which have potential for biased perspectives, systematic reviews utilize pre-determined registered protocols to quantify and summarize the current understanding on a new field of research and uncover areas that require more in-depth analysis.²⁸² By adhering to established eligibility criteria, predefining outcomes and incorporating multiple independent reviewers, systematic reviews minimize bias and increase reproducibility. Our group published a similar investigation in 2015. At that time, we identified only 17 interventional animal studies involving MSC-derived EVs, which did not enable a comprehensive analysis of experimental approach or general efficacy.²¹³ Now, five years later, published research in this field has expanded significantly. This study provides a detailed synthesis on current evidence, methodology, study design and outcomes in EV therapeutics and evaluates their consistency with the MISEV 2014 and 2018 guidelines. The mechanistic pathways and specific biological factors proven to mediate the beneficial effects of MSC-EVs were also analyzed. Results from this systematic analysis will help guide design of preclinical studies with goals of producing robust evidence for future clinical translation of MSC-EVs as a cell-free therapy.

3.3 Research Objectives

We reviewed and evaluated *in vivo* preclinical studies using MSC-derived EVs as an intervention for any animal model of disease or injury. Specifically, we assessed the methodology used in these animal experiments including: (1) interventional characteristics, (2) methods of EV isolation and characterization, (3) experimental design, and (4) EV modifications. As well, we summarized outcomes to evaluate whether MSC-EVs are efficacious and, subsequently, which disease domains may benefit most from this therapy.

3.4 Results / Discussion

3.4.1 Study Characteristics

Applying a search strategy designed by an information specialist, a systematic search of MEDLINE® and Embase databases yielded a total of 754 records. After level 1 screening of titles and abstracts in duplicate, 523 records did not meet our eligibility criteria and were removed. The remaining 231 articles advanced to level 2 full-text screening. Another 25 articles were excluded to generate a final 206 articles that met our eligibility criteria for data extraction and analysis (**Figure 3.1**). A reference list for all 206 included studies can be found in Supplemental File B1. The first published report investigating MSC-derived EVs as an intervention was published in 2009. This was the only article published that year and assessed the therapeutic potential of “microvesicles” in a mouse model of acute kidney injury.³¹⁹ In 2017, 70 articles were published highlighting the increase in research interest for evaluating the therapeutic potential of MSC-derived EVs (**Figure 3.2**). The largest proportion of studies were conducted in China (N=93; 45%), the United States (N=47; 23%), Italy (N=10; 5%), South Korea (N=9; 4%), and Japan (N=8; 4%). A majority of these studies received funding from only academic sources (N=170; 83%), whereas

17 studies (8%) received funding from both academic and private sources, and 2 studies (1%) received funding from only private sources. Seventeen studies (8%) did not report funding sources. Supplemental Files B2 and B3 contain tables detailing individual characteristics including year of publication, country of origin, intervention details, animal model used, and outcomes for each of the 206 included studies.

3.4.2 Experimental Model and Study Design

Over 90% of studies used rodent species (113 mouse studies, 85 rat studies). A small number of papers examined the effects of MSC-EVs in porcine (N=4; 2%), rabbit (N=2; 1%), dog (N=1; 0.5%) or sheep (N=1; 0.5%) models, with no studies using non-human primates. MSC-derived EVs are being explored in a variety of diseases and conditions (**Figure 3.3**). Renal pathologies (N=32; 16%) have been most commonly studied with a major focus on treatment of acute kidney injury. A variety of neurological conditions (N=25; 12%) have also been explored including ischemic or hemorrhagic stroke, traumatic brain injury, and cognitive impairment models. Twenty-one articles (10%) treated musculoskeletal conditions through intra-articular injections of MSC-EVs in models of rheumatoid arthritis or osteoarthritis, or EV-infused scaffolds for bone regeneration. All 20 cardiac studies (10%) focused on ischemic injury established by either temporary ischemia or permanent ligation of a coronary artery. Seventeen articles (8%) investigated the regenerative capacity of MSC-derived EVs for accelerated and scarless wound healing. With the liver (N=16; 8%) and lungs (N=16; 8%) being two common distribution sites for EVs,^{280,320,321} treatment of both acute (*e.g.* acute lung injury or hepatotoxicity) and chronic (*e.g.* pulmonary hypertension or autoimmune hepatitis) states within each domain have been assessed. Fourteen studies (7%) assessed whether MSC-derived EVs promote or inhibit tumor growth.

Seven studies (3%) highlight the role of EVs in completely distinct oculopathies including retinal laser injury, glaucoma, or autoimmune uveitis.

3.4.3 Methods of Isolation

Method of EV isolation has been an area of great debate with no current gold standard. With specific focus on EVs derived from MSC-conditioned media, differential ultracentrifugation was the most widely described method (N=144; 70%) (**Figure 3.4**). Application of manufactured isolation kits was the second most commonly applied method for EV isolation (N=48; 23%) despite marked differences in yield and purity. Ultracentrifugation is known for generating EVs of higher specificity (*e.g.* different sizes of EVs can be isolated by varying spin speeds and duration), but at the cost of lower yield.³²²⁻³²⁴ Conversely, precipitation kits are recognized as a quick approach for producing EVs at much higher recovery but with potentially greater impurities.^{238,322,325} Similar findings were seen in a worldwide survey where more than 80% of EV researchers used centrifugation as their primary isolation technique.²³⁵ Although most popular, the generated centrifugal force and shear stress have been implicated to cause exosome aggregation and potentially alter morphology and function.^{236,238} More importantly, the inefficient yield by ultracentrifugation creates a substantial limitation for clinical scaleup.

One technique that has gained recent interest is the tangential flow filtration (TFF) system. TFF utilizes crossflow filtration and transmembrane permeation of fluids to create a purified EV population. This method allows for adjustment of flow rate to eliminate sedimentation stress, and is less limited by maximum volumes, thereby enabling large quantities of media to be filtered. A recent study demonstrated that TFF improved yield of small EVs by 27-fold compared to ultracentrifugation and may isolate particles of greater potency.²⁴³ Our findings demonstrate that only a handful of published animal studies (N=9; 4%) used TFF for purification by membrane

permeation. Further research is required to compare purity, molecular markers and *in vivo* efficacy of these TFF-enriched EVs to other more commonly used methods. Twenty-two studies (11%) incorporated ultrafiltration of media, many of which preceded the use of a subsequent technique. Twelve studies (6%) used size-exclusion chromatography to enrich their EV population. A variety of other methods were applied as a standalone approach or in combination with other techniques (**Figure 3.4**). Changes in method of isolation over time can be observed in Supplemental File B8. Eight articles (4%) did not provide descriptions regarding isolation technique, which is a crucial detail to determine subtype (*e.g.* small or large EVs) and purity. After isolation, 10 studies (5%) administered EVs immediately or from a refrigerated state, whereas 12 (6%) and 85 (41%) studies froze their product for later use at either -20°C or -80°C, respectively. No description of EV storage condition was provided in 103 studies (50%).

3.4.4 Methods of Characterization

Approaches used to characterize MSC-EVs were even more variable than method of isolation (**Figure 3.4**). As endorsed in the MISEV 2018 document,²²⁷ EVs should be characterized by amount (*e.g.* protein or particle concentration), at least 3 positive and 1 negative protein markers, and 2 measures of single vesicle analysis (including electron or atomic force microscopy, or non high resolution imaging techniques).²²⁷ Only 4 published studies (2%) have met these recommendations for describing their therapy. If we included studies that may or may not have used negative markers but still reported at least 3 positive protein indicators, 29 (14%) met this standard. Lastly, for studies that evaluated expression of at least 1 positive marker with or without negative controls, 61 articles (30%) met these less stringent criteria. It is important to note that many included articles were published prior to the 2018 recommendations. Hence, studies were also assessed for their consistency with the MISEV 2014 guidelines.³²⁶ In these previous

guidelines, the ISEV suggested characterization by at least 2 positive and 1 negative protein markers, and 2 measures of single vesicle analysis, with no recommendations for quantitation.³²⁶ Twelve studies (6%) were found to satisfy these criteria.

Looking at each category individually, single vesicle analysis by electron microscopy (*e.g.* scanning, transmission, cryogenic, *etc.*) or atomic force microscopy were detailed in 69% of studies (N=142), whereas confocal microscopy was used in 6 studies (3%). Quantitation by particle concentration was determined by nanoparticle tracking analysis (N=74; 36%) or tunable resistive pulse sensing (N=16; 8%) (**Figure 3.4**). Furthermore, 137 studies (67%) quantified total protein amount by Bradford/BCA assay as either an independent or supplemental method. Despite many articles providing both quantitative values, a minimal number of studies reported ratios (*e.g.* protein:particle, lipid:particle, or lipid:protein) to estimate product purity as recommended by the ISEV. When assessing protein marker expression, a majority used Western Blot analysis (N=123; 60%) or flow cytometry (N=59; 29%). A minimal number of studies selected other approaches for protein analysis (ELISA kits (N=8; 4%), proteomics (N=5; 2%) or immunofluorescence (N=3; 1%)). As per the specific markers selected, the tetraspanin family of surface proteins including CD63 (N=129; 63%), CD9 (N=99; 48%) and CD81 (N=80; 39%) were the most prevalent. However, choice of protein markers remains highly variable with 28 (14%) and 22 (11%) studies opting to use TSG101 and Alix, respectively, which are protein components of the endosomal sorting complexes. Importantly, 41 studies (20%) did not provide details of whether any specific markers were assessed to ascertain their EV enrichment.

Both the MISEV 2014 and 2018 consensus statements deem it is imperative to examine negative protein markers (*i.e.* non-vesicular proteins derived from the mitochondria, endoplasmic reticulum, Golgi apparatus or nucleus) to demonstrate specific isolation of EVs. However, 90% of

studies (N=185) did not provide descriptions of negative protein controls (**Figure 3.4**). Hence, the molecular mechanism of action behind MSC-derived EVs can be confounded by non-specific cellular components within the isolated product. For studies that did report in accordance with the MISEV guidelines, calnexin (a chaperone protein of the endoplasmic reticulum; N=7, 3%), GM130 (a Golgi membrane protein; N=6, 3%), cytochrome C (a mitochondrial membrane protein; N=5, 2%), tubulin (a cytoskeletal protein; N=4, 2%), or apolipoproteins (N=2; 1%) were used.

3.4.5 Terminology of Extracellular Vesicles

Over the past two decades of EV research, our understanding of EV biology has advanced significantly, thereby necessitating similar alterations in nomenclature. From the included animal studies, 125 (61%) termed their therapy as “exosomes” (**Figure 3.5A**). However, 17 of these studies (14%) did not provide expression analysis of “exosomal” markers (*e.g.* CD9, CD63, CD81, Alix, TSG101, HSP70, or HSP90). Another 54 studies (26%) described their intervention more generally by the term “extracellular vesicles”, 31 studies (15%) as “microvesicles”, 5 studies (2%) as “microparticles”, and 2 studies (1%) as “small EVs”. No papers used the terminology “medium/large EVs”. When assessing the actual size of EVs reported by authors, 42 studies (20%) described a size of 30-1000nm (*i.e.* both small and medium/large EVs), 107 studies (52%) of 30-150nm, 7 studies (3%) of 150-1000nm, and 3 studies (1%) reported using both small and medium/large EVs in separate experiments (**Figure 3.5B**). A quarter of studies (N=51; 25%) did not provide descriptions detailing particle size. Since small and medium/large EVs may vary in their therapeutic effects and bioactive cargo,³²⁷⁻³²⁹ evaluating size is critically important to identify the subtype of EVs being administered as therapy.

As a next step, we sought to determine whether authors used adequate nomenclature for their therapy based on the generally accepted size criteria for different EV subtypes (*e.g.*

“exosomes” or small EVs ~30-150nm; microvesicles or medium/large EVs ~150-1000nm). Sixty-one percent of studies (N=125) were found to describe their EVs consistent with these size definitions (**Figure 3.5C**). For studies that used the term “exosome” and reported a size within the small EV range (N=92), 89 (97%) demonstrated expression of at least one “exosomal” protein in their EV product. The other 3 articles provided no findings of EV marker expression. Hence, we may not be certain that their intervention is in fact “exosomes” of endosomal origin. Thirty-nine percent of papers either did not analyze EV particle size distribution (N=48) or used nomenclature incongruent with their reported particle size distribution (N=35).

3.4.6 Interventional Traits of MSC-Derived EVs

MSCs can be derived from a variety of different sources. Although the majority of studies used bone marrow derived MSCs (N=103; 50%) (**Figure 3.6**), both umbilical cord/Wharton’s Jelly (N=36; 18%) and adipose tissue (N=24; 12%) were also used by a number of investigations, perhaps reflecting the relative ease of collecting these tissues.³³⁰ A number of other tissue sources for MSCs have also been studied including embryonic stem cells-derived MSCs (N=12; 6%), MSCs differentiated from induced pluripotent stem cells (N=8; 4%), synovial membrane (N=4; 2%), umbilical cord blood (N=3; 1%), gingival tissue (N=3; 1%), and renal tissue (N=3; 1%).

Species source of MSCs varied as well and included human (N=128; 62%), mouse (N=41; 20%), rat (N=32; 16%), pig (N=5; 2%), rabbit (N=2; 1%) and dog (N=1; 0.5%) (**Figure 3.6**). Xenogeneic administration of EVs (*e.g.* a human source of MSC-EVs into animal models) were reported in 136 studies (66%), a majority of which demonstrated beneficial outcomes to reflect the immunocompatibility of EVs. This quality is important for future clinical translation as autologous application may not be required. Allogeneic administration was described in 70 studies (34%) and only 2 studies (1%) used autologous transplantation of MSC-EVs, one for canine wound healing

and the other for chronic kidney disease induced in pigs.^{331,332} Three studies (1%) did not provide adequate descriptions to determine EV immunocompatibility.

3.4.7 Dosage Regiment of MSC-EVs

To inform potential future clinical translation, we summarized parameters of EV delivery. The route of administration was highly variable. Nearly half of the studies administered EVs by intravenous injections (N=68 by tail vein, N=25 at peripheral sites and N=4 by jugular vein) (**Figure 3.7**). Other routes included direct tissue injections (N=40; 19%, *e.g.* intramyocardial), subcutaneous treatments (N=26; 13%, the majority of which were for wound healing experiments), and intraperitoneal injections (N=12; 6%). Related to EV modifications, 11 studies (5%) infused EVs onto scaffolds for implantation. Six studies (3%) applied an intratracheal route of administration for efficient lung distribution in models of respiratory injury. Three studies (2%) did not provide any details on route of administration, which is an important parameter in dictating EV pharmacokinetics (*e.g.* bioavailability and distribution). Furthermore, less than a quarter of studies (N=50) performed *in vivo* biodistribution experiments. These investigations are required to ascertain whether EVs reach the target tissue of interest, or if they have pleiotropic effects by influencing multiple tissues and organs. New technologies have been developed to help elucidate the pharmacokinetics of EVs including fluorescent membrane labels for live or post-mortem histological analysis.^{292,321,333,334} Hence, incomplete understanding of how much (if at all) of EVs are reaching the target tissue of interest can hinder interpretation of results and impair future translation.

Over two-thirds of studies (N=139; 67%) injected a single dose of MSC-EVs in their animal experiments whereas 58 studies (28%) utilized multiple doses (**Figure 3.7**). For multiple administrations, 2 (N=20; 10%), 3 (N=18; 9%) or 4 (N=10; 5%) doses were most frequently

applied with many other studies injecting 5 or more doses (Supplemental File B4). Data regarding the timing of administration for multidose experiments can be found in Supplemental File B4. Nine studies (4%) incorporated both single and multiple/repeated dose treatment protocols in their study design. Two articles (1%) provided no description as to whether a single or multi dose regimen was applied. The most heterogeneous parameter for treatment regimen remains to be amount and units of dose. Many studies opted to dose their EVs by absolute protein amount (*e.g.* µg of EVs) (N=139; 67%) or by particle concentration (N=32; 16%)—both representing very distinct measures of quantification. For individualized protocols, some groups dosed by the amount of protein (N=9; 4%) or particles (N=2; 1%) per weight of the animal. Even more complex were studies that determined dosages by original cell count of MSCs (*e.g.* the amount of EVs made by one million MSCs in culture) (N=9; 4%) or by the amount of EVs made over time in cell culture (*e.g.* total EVs made over 48h of MSC proliferation) (N=6; 3%). Among those who used protein or particle counts, the amount delivered was highly variable. The exact dose of MSC-EVs delivered for each study is outlined in Supplemental File B4. Twelve studies (6%) provided no description on the dosage of EVs used. Notably, few studies (N=29; 15%) incorporated dose-response studies. Of the studies that did conduct dose analyses, 14 (48%) evaluated two different doses, 10 (34%) evaluated three doses, 3 (10%) evaluated four doses and 2 (7%) evaluated six doses. The exact doses or range of doses for each study can be found in Supplemental File B4.

With regard to timing of EV therapy, 185 studies (90%) conducted a ‘rescue’ protocol whereby MSC-EVs were administered post disease induction (**Figure 3.7**). Thirteen studies (6%) delivered EVs prior to established injury as a preventative therapy. Eight papers (4%) injected therapy concurrently with disease induction and 9 papers (4%) assessed both prevention and treatment protocols.

3.4.8 Modifications of MSC-Derived EVs

As engineered cell therapies have become more prominent in both preclinical and clinical studies, we evaluated the prevalence of EV modifications including changes to protein expression, knockdown/augmented microRNA packaging or administration of EVs with other biomaterials (*e.g.* scaffolds, gels, *etc.*). A majority of papers included administration of MSC-EVs without any modifications (N=176; 85%) (**Figure 3.8**). Ninety-six articles (47%) evaluated the impact of modifications or co-treatment of MSC-EVs for a total of 124 distinct studies. Of these 124 studies, 83 altered source MSCs before EV isolation (67%), 37 modified EVs directly (30%) and 23 studies involved co-injection of EVs (19%) (*e.g.* with cells, pharmacologic therapies, or inhibitors).

Forty-nine studies utilized MSC transfection/transduction to induce overexpression (N=23) or knockdown gene expression with shRNA/siRNA (N=24) (**Figure 3.8**). Of the 49 studies, 20 altered the packaging of specific microRNAs into EVs (N=5 for knockdown, N=15 for overexpression). Twenty-five studies genetically modified protein expression to enhance EV efficacy (N=8) or investigated mechanism of action through downregulation (N=17). Interestingly, the 14-3-3 ζ protein was shown to be a key regulator for stem cell expansion and collagen deposition. Knockdown of its expression abolished the therapeutics effects of EVs for cutaneous wound healing.³³⁵ In a separate study, the importance of 14-3-3 ζ was further confirmed when its overexpression enhanced EV efficacy by activating cellular autophagy, promoting proliferation and reducing apoptosis to prevent nephrotoxicity.³³⁶ One study overexpressed hypoxia inducible factor 1 alpha (HIF1 α) to accelerate angiogenesis *in vivo* and 2 studies generated MSC-EVs expressing a mutated, nondegradable form of HIF1 α which promoted bone/joint regeneration.³³⁷ Two studies investigated the effects of altering Argonaute-2 expression, an essential protein of the RNA-induced silencing complex required for binding and cleavage of target mRNAs by

microRNAs.^{338,339} Attenuated Argonaute-2 by siRNAs in MSCs allowed for global downregulation and dysfunction of microRNA in EVs to their impact. Lastly, an important issue with EV production is the limitation in MSC passaging and expansion. One study addressed this issue by transfecting MSCs derived from human embryonic stem cells with the Myc gene to produce an immortalized cell population.³⁴⁰ “Exosomes” from these MSCs reduced infarct size in a murine model of myocardial infarction. Hence, Myc transformation represents a possibility to generate an infinite source of EV production which is needed for clinical scaleup.

Thirty-six studies explored pre-treating either conditioned media or EVs prior to their administration (**Figure 3.8**). A majority utilized broad enzymatic digestion (N=13 for RNase, N=5 for proteinases) to determine whether RNA or protein packaging may be of greater importance for EV efficacy. Twelve studies incorporated EVs onto scaffolds prior to their implantation with one study resuspending EVs in polyethyleneimine to augment scaffold adhesion. Five studies incubated neutralizing antibodies or shRNA with EVs. One utilized soluble hyaluronic acid to reduce EV incorporation, whereas Tamura *et al.* (2017) modified “exosome” surface membranes with cationized pullulan to enhance biodistribution and immunomodulatory effects in a mouse model of liver injury.³⁴¹ Co-treatment of EVs with other components (*e.g.* inhibitors, cells, antibodies, *etc.*) were applied in 17 studies (Supplemental File B5).

Twenty-nine studies preconditioned MSCs prior to media collection and EV isolation (**Figure 3.8**). Of these studies, 12 cultured MSCs under hypoxic conditions (N=1 at 0% O₂, N=1 at 0.5% O₂, N=5 at 1% O₂, N=2 with cycles of anoxia/reoxygenation, and N=2 did not specify degree of hypoxia). Hypoxic conditioning of MSCs is known to augment secretion of EVs enriched with protective factors *in vitro*. Accordingly, 5 of 6 studies that compared *in vivo* outcomes between hypoxic and normoxic conditions demonstrated improved efficacy from administration

of hypoxia derived EVs. Three studies found improved functional outcomes (N=1 for spatial learning/memory in a model of cognitive impairment, 0% O₂; N=1 for survival of fat graft, 5% O₂; N=1 for heart function, 0.5% O₂), one of which also showed prolonged survival in a mouse model of myocardial infarction. However, EVs derived from hypoxic conditioning of adipose MSCs (1% O₂) were inferior to normoxic EVs for alleviating the systemic inflammatory response, respiratory insult or renal injury associated with preclinical septic shock.³⁴² Much like hypoxic preconditioning, culturing MSCs with pro-inflammatory factors are believed to induce an anti-inflammatory gene response in MSCs, thereby leading to increased packaging of anti-inflammatory mediators in EVs. From our findings, 5 studies cultured MSCs with pro-inflammatory cytokines (*e.g.* TNF α , IL-1 β or IFN- γ), 3 studies with lipopolysaccharide (toll-like receptor 4 agonist), and 2 with polyinosinic:polycytidylic acid (Poly I:C; toll-like receptor 3 agonist). EVs derived from pro-inflammatory preconditioning demonstrated greater efficacy than unmodified EVs for *in vivo* outcomes in 5 studies, including improved locomotor function after spinal cord injury and increasing survival during sepsis.^{232,343} Conversely, two studies demonstrated contradictory findings whereby LPS or IFN- γ conditioning produced EVs that were markedly less effective at restoring nerve or kidney function, respectively.^{344,345} Further studies are needed to determine the ideal duration/concentration of hypoxia and pro-inflammatory preconditioning to maximize EV yield and effectiveness. Other approaches for preconditioning MSCs include co-culturing (N=2), hormone additives (N=1), herbal extract (N=1), brain tissue extract (N=1), culturing on scaffolds (N=1), or supplementing media with nitric oxide polymers (N=1) (**Figure 3.8**).

3.4.9 Molecular Targets and Mechanistic Pathways Targeted by MSC-EVs

There have been 113 published studies that either enhanced EVs (N=53; 47%) or explored their mechanism of action (N=60; 53%) (**Figure 3.9A**). A majority focused on specific proteins (N=34; 30%) or microRNA (N=23; 20%) targets by downregulating their expression in MSCs. Furthermore, 16 studies utilized inhibitors/antagonists, 2 studies created a knockout mouse model of miR-21a and miR-223, and one study knocked out specificity protein 1 (SP1) in iPSCs before differentiating into MSCs. From these modification studies, we synthesized their data to gain a better understanding of the mechanistic pathways behind MSC-EV therapeutics. The most common physiological pathways that mediate efficacy were inflammation/immunomodulation (N=38; 34%), apoptosis (N=31; 27%), angiogenesis (N=24; 21%), cell proliferation (N=20; 18%), tumorigenesis (N=13; 12%), fibrosis (N=11; 10%), cell migration (N=6; 5%), regeneration (N=6; 5%), and EV incorporation/biodistribution (N=5; 4%) (**Figure 3.9B**). From heat map analysis, a difference is evident between the pathways targeted by studies of enhancements as compared to EV mechanism (**Figure 3.9B**). EV enhancements most commonly focus on modulating inflammation (N=25/53, 42% for enhancement studies vs. N=13/60, 22% for mechanistic studies) whereas mechanistic studies concentrated on factors affecting apoptosis (N=11/53, 21% for enhancement studies vs. N=20/60, 33% for mechanistic studies) or proliferation (N=2/53, 4% for enhancement studies vs. N=18/60, 30% for mechanistic studies). Comparative studies are needed to determine which of anti-inflammatory, anti-apoptotic, or pro-proliferative factors are most crucial for mediating EV efficacy.

Data concerning specific molecular factors associated with the top 6 mechanistic pathways were analyzed (**Figure 3.10**). *Inflammation*: The microRNAs determined to enhance MSC-EV immunomodulation include miR-223 (improved liver function), miR-146a (improved survival during sepsis), and miR-181 (prevented liver fibrosis and attenuated burn-induced inflammation).

miR-223 and miR-146a in MSC-EVs were further shown to be critical anti-inflammatory components and organ protective from knockdown experiments in preclinical polymicrobial sepsis. With regards to anti-inflammatory proteins, HIF1 α , IL-10, C-C motif chemokine receptor-2 (CCR-2), keratinocyte growth factor, angiopoietin-1, and vascular endothelial growth factor (VEGF) are all possible targets to optimize EV interventions. *Apoptosis*: miR-223 overexpression also attenuated cellular apoptosis, along with miR-21 and miR-133 in rat models of intervertebral disc degeneration and intracerebral hemorrhage, respectively. Overexpression of critical proteins that were found to significantly reduce apoptosis and improve organ function include HIF1 α , C-X-C motif chemokine receptor 4 (CXCR4), GATA-4, and 14-3-3 ζ . VEGF, glutathione peroxidase-1, and miR-30 may also be plausible enhancement targets as their inhibition led to abolished anti-apoptotic effects in bronchopulmonary dysplasia, hepatotoxicity and ischemia-reperfusion renal injury, respectively. *Angiogenesis*: miR-126a overexpressed in synovium-derived MSC-EVs accelerated vascularization and wound closure in diabetic rodents, whereas suppression of miR-210 eliminated the pro-angiogenic and anti-fibrotic quality of EVs. Echoing their anti-apoptotic effects, CXCR4 overexpression and HIF1 α overexpression/non-degradable mutation led to augmented angiogenesis. Increasing exosomal packaging of protein kinase B (also known as Akt), a known regulator of cellular survival, promoted angiogenesis in a model of myocardial infarction which led to a markedly improved left ventricular ejection fraction. Other angiogenic EV proteins determined by knockdown studies include VEGF, extracellular matrix metalloproteinase inducer (EMMPRIN), jagged-1 (a crucial ligand required for HIF1 α function), Wnt4/ β -catenin signalling, and deleted in malignant brain tumors 1 (DMBT1) protein. *Proliferation*: Both Wnt4 signalling and DMBT1 were confirmed to also be critical components for EV-mediated cell proliferation. Augmented levels of miR-140 promoted cell division in rodent osteoarthritis and 14-3-3 ζ

improved kidney function in the setting of cisplatin-induced nephrotoxicity. *Fibrosis*: miR-181 or GATA-4 overexpression in MSC-EVs attenuated CCl₄-induced liver fibrosis and cardiac fibrosis, respectively. Moreover, the anti-fibrotic effects of EVs were aberrated after downregulation of miR-210 or miR-22 in permanent ligation models of myocardial infarction. *Tumorigenesis*: Targeted delivery of shRNA for oncogenic KRAS into “exosomes” attenuated tumor growth and markedly improved survival in multiple models of pancreatic cancer, in part due to CD47-mediated evasion of host immune cells.³⁴⁶ Administration of bone marrow-derived MSC-EVs overexpressing miR-146b and miR-379 resulted in reduced tumor growth for malignant glioma and breast cancer, respectively. Although increasing levels of miR-122 in EVs did not result in efficacy on its own, miR-122 induced a heightened sensitivity to the chemotherapy sorafenib. Hence, a co-treatment approach led to significantly suppressed tumor progression. Lastly, MSC-EVs enriched with miR-124a was the only treatment to exhibit survival benefits in cancer studies. Fifty percent of mice were alive at 110 days. Histological analyses of brain tissue for glioblastoma demonstrated a complete regression of tumors, indicating that mice were cured by the genetically enhanced MSC-EVs.³⁴⁷

3.4.10 General Outcomes and Benefits from MSC-EV Modifications

Based on statistical significance in preclinical outcomes, studies were categorized as demonstrating “beneficial”, “mixed benefits” or “no benefits” from EV administration. Significant improvements of all reported outcomes in animals treated with MSC-EVs were reported in 166 studies (81%) (**Figure 3.11A**). Another 33 studies (16%) reported benefits in at least one (but not all) of their findings, and 4 studies (2%) showed null effects from the delivery of MSC-EVs. Three studies (2%) reported adverse effects. One of these articles suggested that “exosomes” released by islet-derived MSCs may carry autoantigens to trigger activation of autoreactive B and T cells

involved in destruction of insulin-producing β -cells.³⁴⁸ To investigate the role of EVs in tumor progression, two studies injected EVs intratumorally in xenograft mouse models of human renal cell carcinoma³⁴⁹ or nasopharyngeal carcinoma.³⁵⁰ A marked acceleration in tumor development and size was observed which was attributed to vesicular transfer of mRNA (*e.g.* hepatocyte growth factor)³⁴⁹ or peptide (*e.g.* fibroblast growth factor).³⁵⁰ More research is required to discern whether MSC-derived EVs are truly beneficial or detrimental in the setting of cancer.

Over 40% of the modification studies (N=53) were designed with intention of enhancing therapeutic efficacy. Studies were categorized based on the proportion of *in vivo* outcomes that were statistically improved from administration of modified MSC-EVs as compared to unmodified MSC-EVs (**Figure 3.11B**). These categories included: (1) improvements observed for all *in vivo* outcomes (N=18; 34%), (2) most *in vivo* outcomes ($\geq 50\%$ of reported findings) (N=13; 25%), (3) some *in vivo* outcomes ($< 50\%$ of reported findings), (N=7; 13%), (4) equal efficacy (N=2; 4%), (5) worse outcomes with modified EVs (N=3; 6%), or (6) unclear / no comparative studies (N=10; 19%). Functional data was also extracted and 21 studies exhibited markedly improved organ function after treatment with enhanced EVs including attenuated liver enzymes, reduced serum creatinine or blood urea nitrogen in kidney injury, accelerated wound healing, improved cardiac contractility, and suppressed tumor growth. Five studies also demonstrated significant survival benefits from enhanced EVs compared to unmodified EVs. Importantly, 5 studies that reported improvements to most *in vivo* findings did not observe the same benefits from EV modifications when evaluating functional outcomes, although still significantly improved when compared to vehicle control. Lastly, 3 studies observed worse functional data upon administration of altered EVs as compared to control EVs. Two of these studies attempted to precondition MSCs with IFN-

γ or LPS and one study cultured MSCs under 1% oxygen for 12 hours which reduced survival benefits in the setting of sepsis.

Despite a majority of studies reporting beneficial outcomes, potential sources of bias were recognized in many reports. With regards to bias intrinsic to the study design, only 42% (N=87) of studies incorporated randomization. Furthermore, the overwhelmingly positive findings (97% of articles reported significant benefits in at least one outcome) suggest publication bias. Our study indicates that only 1.9% (N=4) of studies demonstrated null effects from administration of MSC-EVs. These results are similarly observed in a previous study that shows publication bias to be highly prevalent in animal experiments of stroke (1.2% reported no significant benefits).³⁵¹

There are limitations in our methodological design that we hope to address in future studies. First, we have categorized outcomes by complete, mixed or null benefits. It was initially planned to conduct a meta-analysis of primary outcomes. However, the number of included full-text articles after screening was significantly larger than the last systematic review²¹³ and varied across numerous disease states. Hence, we decided to first focus on experimental approach, study design and mechanism of action behind EV therapy. Studies have been subcategorized by disease domain and future meta-analyses within each domain will quantitatively evaluate the conditions that may benefit most from MSC-EVs. In addition, subgroup analyses by methodology variables (*e.g.* species/tissue source of MSCs, culture conditions, isolation technique, EV subtypes, modifications, dose, disease) will be conducted to elucidate the optimal methods for EV efficacy. A second limitation is the lack of assessment on risk of bias (aside from randomization) or quality of reporting. It has become apparent that rigorous methodology in preclinical study design is lacking, which may be a source for the poor translation of promising therapies from bench to

bedside.³⁵²⁻³⁵⁴ Future systematic reviews will look closely at important factors that affect bias and quality of reporting.³¹⁰

3.5 Conclusions

Our systematic review highlights a growing interest to develop MSC-derived EV therapy. However, nomenclature, experimental approach for isolation and characterization, interventional traits and treatment protocols were found to be highly heterogeneous. Despite this variability, benefit across all measured outcomes were reported in 81% of studies and improvements in at least one outcome in 97% of studies. Compared to unmodified MSC-EVs, EV enhancements led to improved outcomes, organ function and survival in 72% of studies. Moreover, benefits from this cell-free therapy are illustrated in diseases spanning all organ systems with completely disparate pathophysiology. These findings suggest that MSC-derived EVs have the potential to treat conditions across a variety of diseases with minimal adverse effects.

As with any new field of research, a large number of narrative reviews have been recently published to discuss the therapeutic potential of MSC-derived EVs. Many of these articles focus on pre-selected studies and preferred topics thereby increasing susceptibility to bias unless careful examination of all available information is considered.²⁸¹⁻²⁸³ A recently published narrative review summarized current trends in preclinical experimentation of MSC-derived EVs, suggesting some of the same heterogeneity observed in our findings.³⁵⁵ In our study, however, we used a methodologically rigorous systematic review approach to develop a detailed analysis on methodology, interventional details, critical pathways/molecular factors and overall efficacy of MSC-EVs in all animal studies published to date. By following gold standard practices of systematic reviews (*e.g.* registering a protocol *a priori* and having pre-defined questions and

outcome assessments) our study provides a robust and objective analysis of EV research. Moreover, this approach has identified opportunities for future development and significant knowledge gaps that require greater attention. First, the exceedingly high rate of positive results indicates a strong publication bias within the field. It is likely that a ‘file-drawer’ effect is at work (*i.e.* negative and null results are stored away and never disseminated); we hope that EV researchers can find forums to share negative results, as this will only help advance the field. In addition, greater standardization of nomenclature is required to permit between study comparisons as different subtypes of EVs (*e.g.* medium/large EVs, small EVs or exosomes) may exhibit distinct therapeutic effects. According to size criteria, 35 articles used inconsistent terminology to describe their therapy. This is likely an underestimate since our criteria for “consistency in terminology” was liberal and did not incorporate methods of isolation or expression of “exosomal” markers. Equally problematic was our finding that 51 articles did not provide details of particle size analysis. Future studies should also consider providing a better understanding of the pharmacokinetics and tissue specific distribution of MSC-EVs. This information will be critical to optimize dosage and delivery. Finally, numerous articles were found to have inadequate or no descriptions for various experimental procedures. Only 12 and 4 studies met the MISEV 2014 and 2018 reporting guidelines, respectively. Compounded with the high heterogeneity in EV research methodology, we encourage more transparent and complete reporting of methods to develop this rapidly growing field. Irreproducible findings in preclinical research are estimated to exceed 50% leading to a substantial economic waste in resources. Inconsistent reporting of experimental design has been attributed as a critical factor.^{288,289,356} The MISEV guideline represents a meaningful way for EV researchers to demonstrate validity in experimental design and detailed methodology for reproducibility. Development of the online EV-TRACK Platform has enabled researchers around

the world to submit their experimental approach and receive a metric on their completeness of reporting.²⁴⁵ The robust *in vivo* findings from our systematic analysis may help create a preclinical extension of EV-TRACK and incorporate information of target molecules and cellular pathways that enhance EV efficacy. Given the abundant choices available when designing animal studies (*e.g.* animal model, species/tissue source of MSCs, techniques for isolation/characterization, dose and many others), improved reporting will facilitate rigorous comparison of techniques necessary to determine the most efficient and effective approaches before investing considerable resources into clinical studies. Greater attention to these issues identified by our systematic analysis may accelerate the translational process of EV therapies.

3.6 Methods / Experimental

3.6.1 Systematic Review Protocol and Registration

Our protocol was registered in the International Prospective Registry of Systematic Reviews (PROSPERO CRD42019123918) and has been published.²²² Reporting of this systematic review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines (see Supplemental File B6).³⁵⁷

3.6.2 Eligibility Criteria

Eligibility criteria were created *a priori* to guide decisions as to which articles would be included for full-text data extraction. Predefining our population, intervention, comparator, outcome and study design (PICOS) parameters is a standard approach applied in systematic reviews to allow for objective, reproducible analysis.

Population: We included any *in vivo* animal model of disease, organ injury, or altered immune response. Studies involving *In vitro*, *ex vivo*, or invertebrate preclinical models were excluded from data extraction.

Intervention: Studies investigating the use of EVs (*e.g.* small or large EVs, exosomes, and/or microvesicles) derived from MSCs of any tissue source were included. Administration of EVs to animal models of disease were xenogeneic, allogeneic or autologous. MSCs or EVs were unmodified, pre-conditioned, modified (*e.g.* gene transfected, protein or microRNA overexpression, *etc.*) or administered as a co-treatment with another therapy. Studies where EVs were not directly administered into animal models or were studied for non-therapeutic effects (*e.g.* EV contribution to tumour growth, biodistribution experiments) were excluded from this systematic review.

Comparator: Preclinical studies with any comparator were included (*e.g.* vehicle control, fibroblasts, fibroblast EVs, *etc.*).

Outcome: Data pertaining to interventional traits (*e.g.* tissue source of MSCs, dosage regimen, modifications, *etc.*), methods of isolation, characterization, experimental design (*e.g.* randomization, biodistribution experiments), and overall direction of all reported *in vivo* outcomes were collected.

Study Design: Any controlled comparison interventional study was included. Experiments were randomized, pseudo-randomized or non-randomized in design. Unpublished grey literature, abstracts, reviews, commentaries and editorials were excluded from this systematic review.^{358,359} Single arm interventional studies (*i.e.* uncontrolled studies) were excluded.

3.6.3 Search Strategy

A search was conducted using the following databases: Ovid MEDLINE®, Ovid MEDLINE® In-Process & Other Non-Indexed Citations, Embase Classic + Embase, and BIOSIS. All search strategies were produced by an information specialist (Risa Shorr, Learning Services, The Ottawa Hospital) with expertise in systematic literature searches. A second information specialist validated the search strategy using Peer Review of Electronic Search Strategies (PRESS).³⁰⁵ Controlled vocabulary, MeSH terms (*e.g.* mesenchymal stem cells, mesenchymal stromal cells, extracellular vesicles, exosomes, microvesicles) and acronyms (*e.g.* MSCs, EVs, MVs) were used and adapted accordingly for different databases. Filters specific for preclinical animal studies were applied. No articles were excluded for language or date of publication. The complete search strategy can be reviewed in Supplemental File B7.

3.6.4 Study Selection Process

All records obtained from the search strategy was uploaded to the Distiller Systematic Review Software (DistillerSR, Evidence Partners, Ottawa, Canada), a program for literature review with enhanced transparency and replicability. For level 1 screening, two independent reviewers examined all titles and abstracts for adherence to the proposed eligibility criteria. For all records that were deemed eligible after level 1 screening, full-text articles were uploaded to DistillerSR for further review. A calibration exercise involving a set of 10 full-text articles was completed to confirm high inter-rater correlation ($kappa > 0.8$) before level 2 full-text screening was conducted in duplicate (AT, MS). Any disagreements between reviewers were resolved by a senior member of the team (MML or DSA). An overview of the study selection process is represented by a PRISMA flow diagram (**Figure 3.1**).

3.6.5 Data Extraction

Standardized data extraction forms were designed by the review team and uploaded onto DistillerSR to facilitate data collection. Before starting data extraction, a calibration exercise between two independent reviewers (AT, MS) from the first 20 studies was completed to ensure consistent data extraction. All information collected was compared between both reviewers and any conflicts were resolved before summarizing the data. Data was collected on general study characteristics (*e.g.* country of corresponding author, year of publication, funding sources, *etc.*) and the following data items: (1) interventional characteristics, (2) methodology used for EV enrichment and characterization, (3) experimental design, (4) EV modifications and (5) treatment outcomes.

3.6.6 Interventional Characteristics

Intervention details include information regarding type of EV administered (*e.g.* small, large or both EVs), animal/tissue source of MSCs, route and number of administrations, dosage units, and timing of treatment. The generally accepted size criteria for EV subtypes (*e.g.* exosomes or small EVs ~30-150nm, microvesicles or medium/large EVs ~150-1000nm) was applied to determine whether authors utilized consistent terminology to describe their therapy. Although the ISEV advises researchers to use broader terms such as small or large EVs, we considered the term “exosomes” to be “consistent” for EVs less than 150nm in diameter since many of these studies may have been published before the MISEV 2018 guidelines. For studies that describe their intervention as “exosomes” but reported a size greater than 150nm, this would be categorized as an inconsistent use of terminology. Articles that used contradictory terms interchangeably (*e.g.* “exosomes” and “microvesicles”) were categorized as an inconsistent use of terminology.

3.6.7 EV Methodology, Intervention Details and Modifications

Data pertaining to experiment methodology included techniques for isolation, approaches to characterization, and choice of selected protein markers. Study design details include disease or condition of interest, type of animal models, treatment/control groups, descriptions of randomization, and biodistribution experiments. Studies involving modified EVs were categorized into modifications before EV isolation (*i.e.* preconditioning or MSCs, transfections/transductions, isolating MSCs from knockout cells, tissues or animals), modifications to EVs directly (*i.e.* preconditioning EV suspension, implanting EVs onto scaffolds), or co-injection of EVs with other agents. The biological target and specific molecular factor for each study was extracted. Studies were subdivided by reason for modification including investigations for EV enhancements or mechanism of action. Lastly the physiological pathway targeted by EV modifications were determined by evaluating all *in vivo* outcomes and reviewing the results/discussion from each article. Cellular processes targeted by EV enhancements were compared to mechanistic studies and represented by a heat map.

3.6.8 Study Outcomes

Study outcomes were determined by thorough assessment of all results and figures. MSC-EVs were deemed as “beneficial” if all reported outcomes showed a statistically significant improvement in the intervention group. “Mixed benefits” were reported for articles with at least one outcome having no significant changes compared to control, whereas “no benefits” were articles reporting no differences in all outcomes when comparing MSC-EVs to control. “Adverse effects” are studies that reported worse outcomes upon MSC-EV administration.

The benefit of EV enhancement was determined by evaluating whether *in vivo* outcomes were statistically improved by modified EVs as compared to unmodified EVs. Studies were categorized by improvements to “all reported outcomes”, “most reported outcomes” ($\geq 50\%$),

“some reported outcomes” (<50%), “equal efficacy”, “worse” if unmodified EVs demonstrated greater efficacy, or “unclear” if studies did not compare modified to unmodified EVs.

All extracted data from included articles are presented as frequencies, proportions or percentages. Studies may have used multiple techniques and interventions, either in separate experiments or in combination. Hence, the cumulative percentage may be greater than 100%, and the number of studies for a given data item (*e.g.* isolation technique) may be greater than the total number of included studies (N=206).

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3.8 Supporting Information

The supporting information is available online free of charge. The supplemental files include extracted data from all 206 studies featured in this systematic review. Supplemental File B1 provides a reference list for all 206 included studies. Supplemental File B2 describes the interventional details, Supplemental File B3 describes outcomes and animal models, Supplemental File B4 details dosage data, and Supplemental B5 describes modification data of MSC-derived EVs. Supplemental File B6 features our PRISMA Checklist and Supplemental File B7 features the search strategy implemented for the systematic literature search. Lastly, Supplemental File B8 indicates the method of EV isolation used per year.

Disclosure Statements

DJS is an advisor to Northern Therapeutics (Montreal, QC, Canada). The remaining authors have no competing interests to declare.

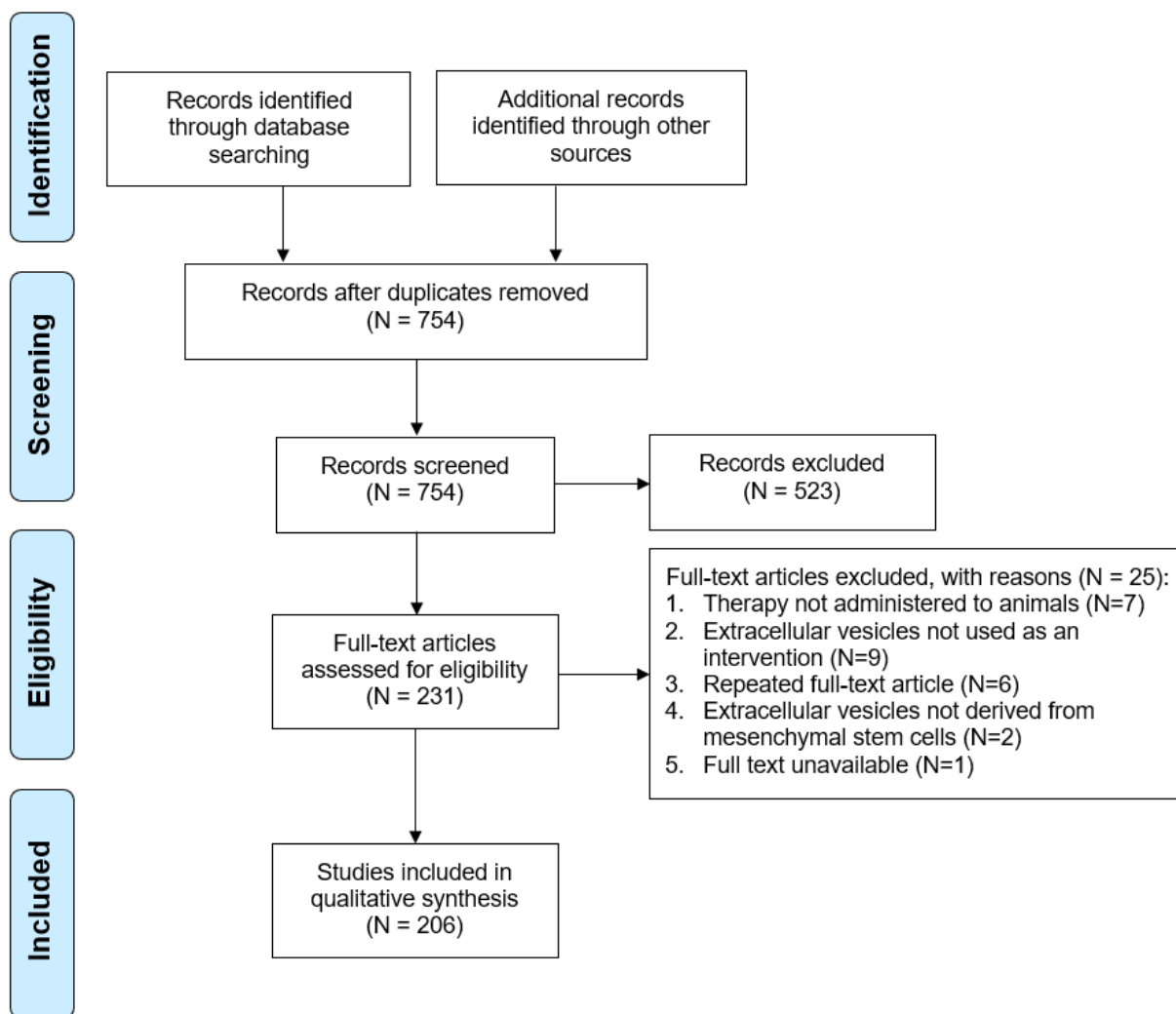


Figure 3.1. Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) flow diagram detailing study screening and selection.

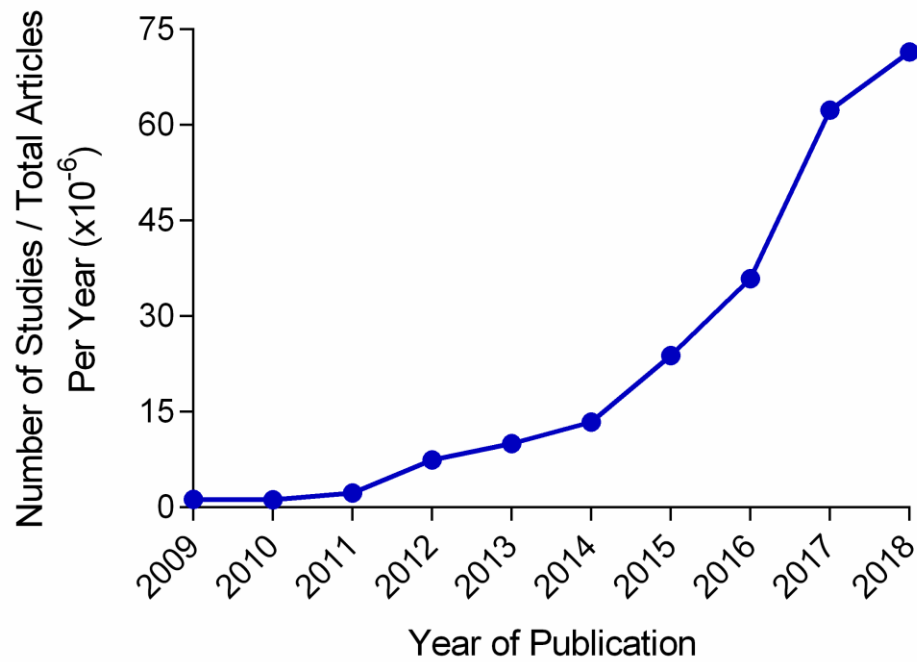


Figure 3.2. Number of preclinical MSC-EV publications.

Number of preclinical animal studies published per year investigating mesenchymal stromal cell-derived extracellular vesicles as a therapy. Values are normalized to total number of PubMed articles published per year. The number of MSC-derived EV studies published in 2018 is extrapolated from the total number of included studies until May 2018 (the end of our search date).

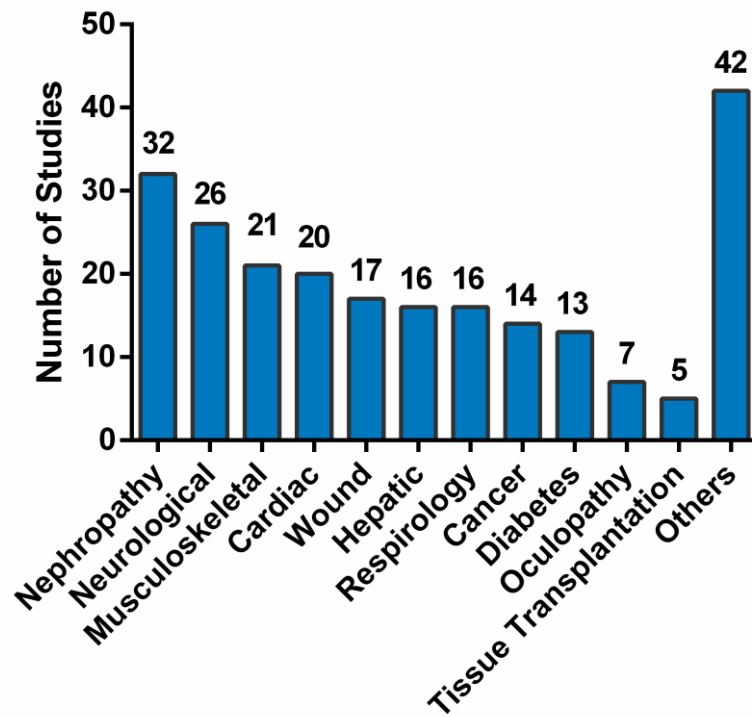


Figure 3.3. Disease domains of preclinical MSC-EV studies.

The disease domains that have been evaluated for therapeutic efficacy of mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs). The category “others” represents a variety of conditions including intervertebral disc degeneration, spinal cord injury, pancreatic disease, hair growth, nerve injury, angiogenesis, ischemia, and others. Articles were included under multiple disease domains if they included more than one condition.

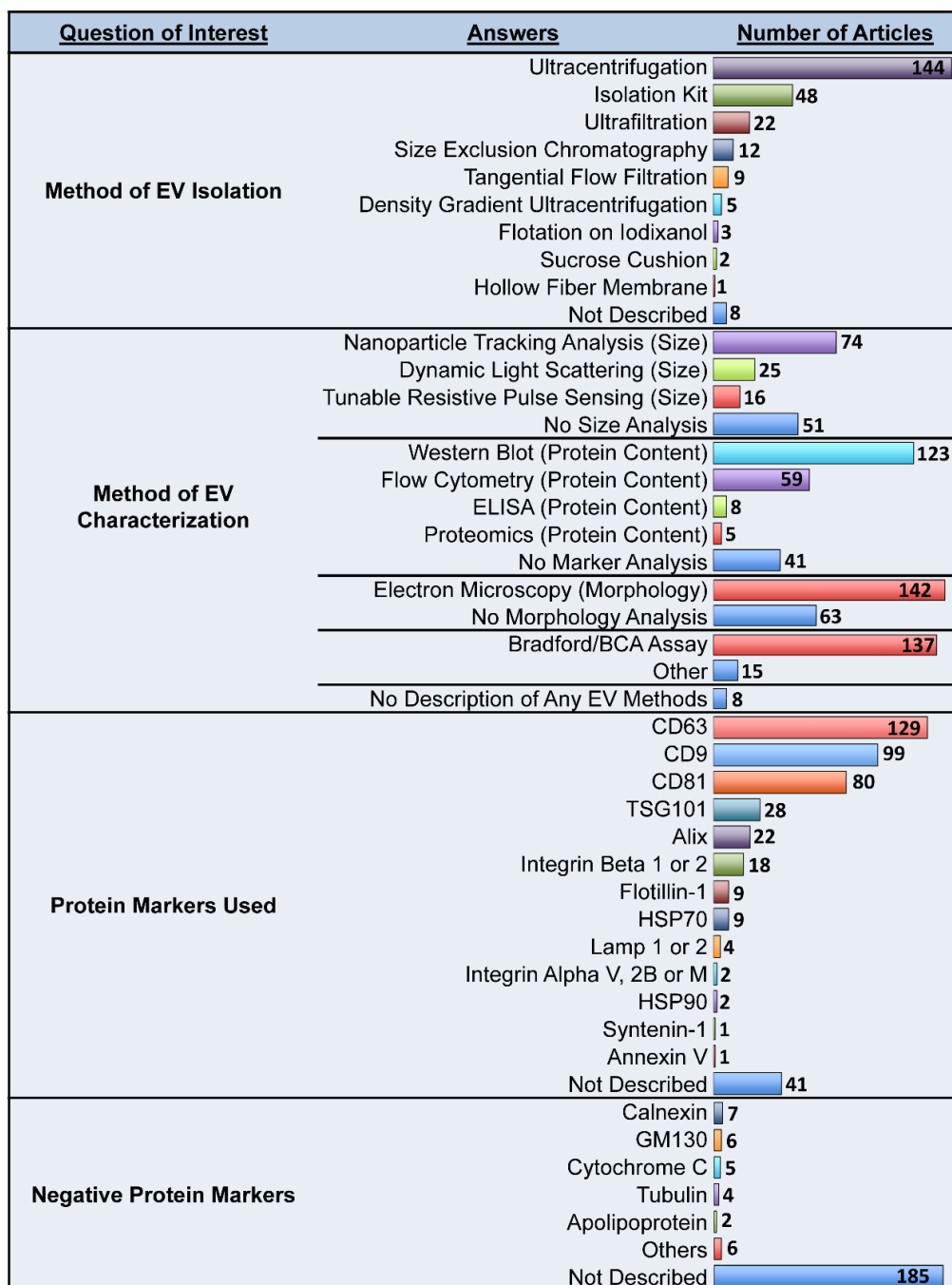


Figure 3.4. Methodology of preclinical MSC-EV studies.

Overview on methods of extracellular vesicle (EV) isolation and characterization from all 206 articles. The specific positive and negative protein markers being used were recorded. Studies could have incorporated more than one method within each domain.

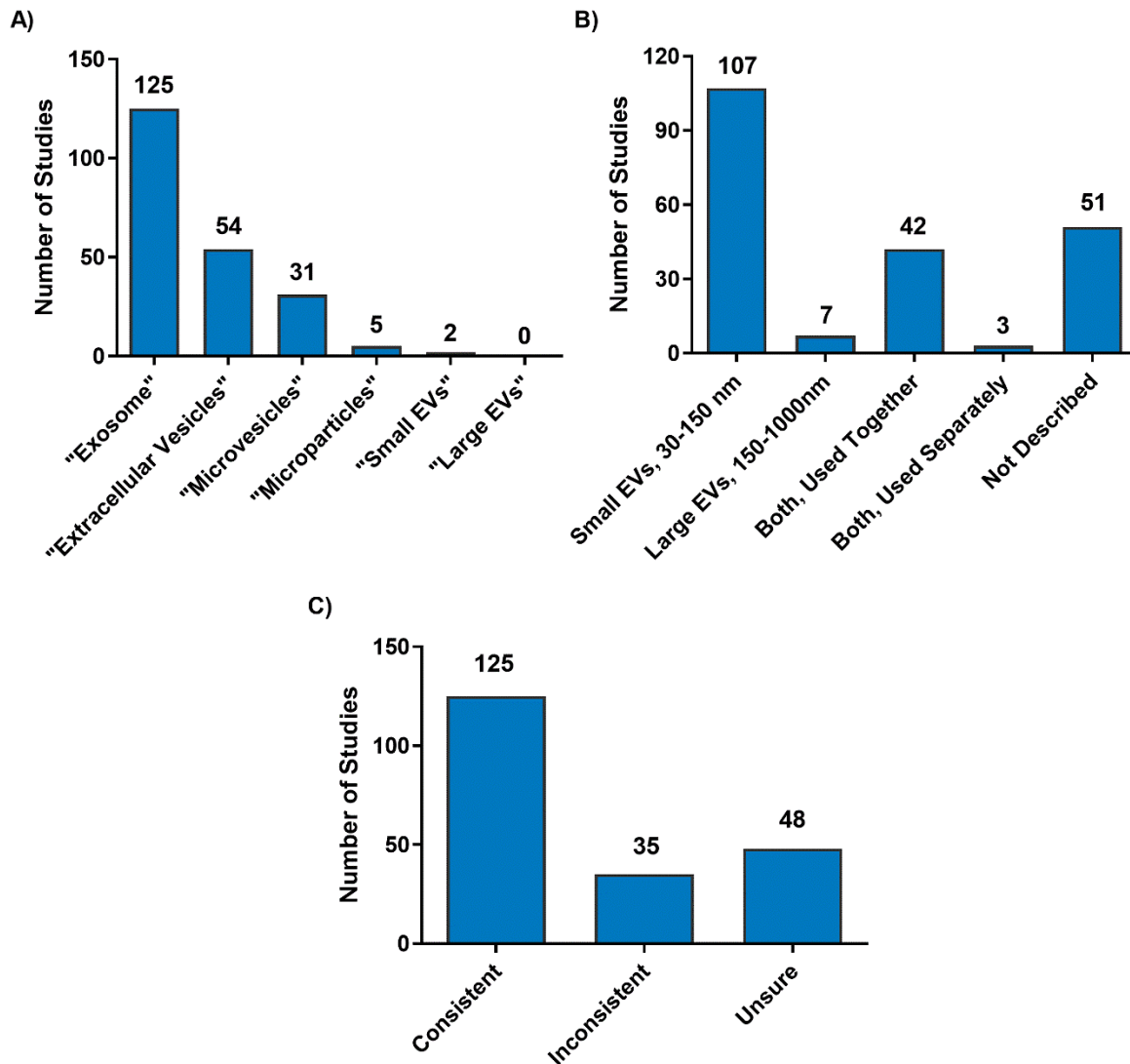


Figure 3.5. Overview on nomenclature from 206 included articles.

(A) The terminology used by authors to describe their extracellular vesicle (EV) therapy was recorded. (B) Size description of EVs was also extracted. (C) Studies were categorized as using terminology “consistent” or “inconsistent” with size distribution (exosomes or small EVs ~30-150nm, and microvesicles or large EVs ~150-1000nm). Forty-eight studies were not able to be categorized for consistency in terminology as no details in EV size were reported. Articles that used terms interchangeably (e.g. “exosomes” and “microvesicles”) were categorized as “inconsistent”.

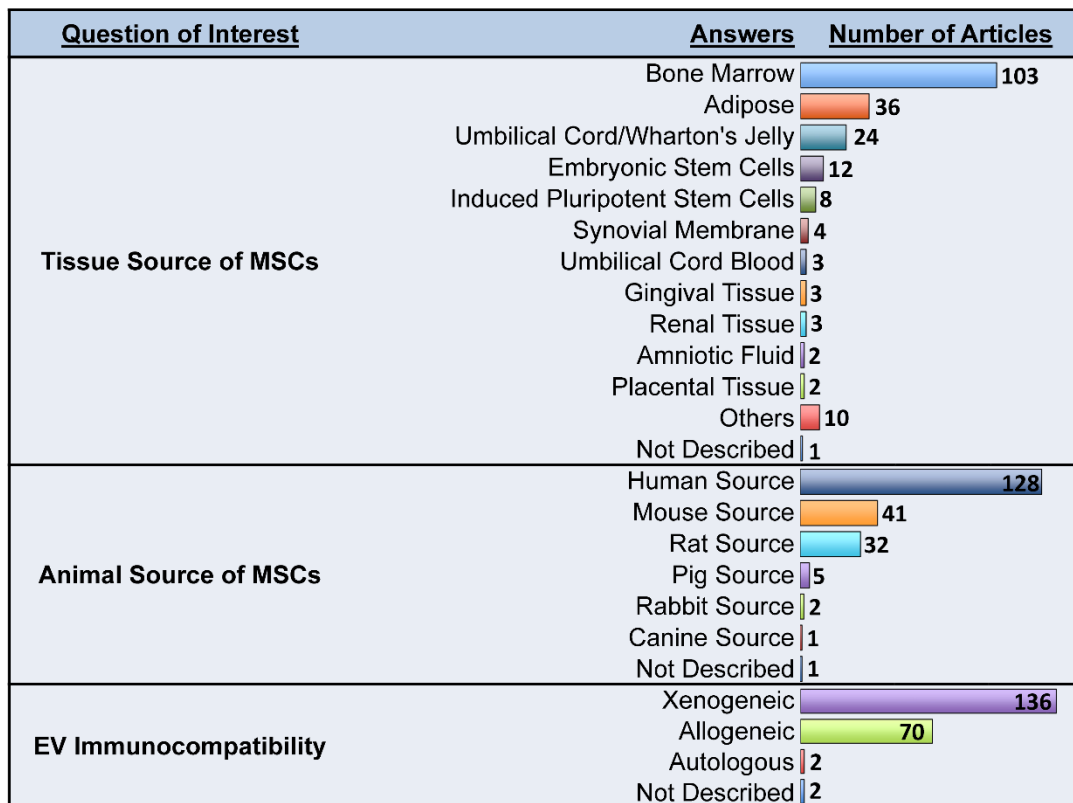


Figure 3.6. Overview of interventional details for administered MSC-EVs.

The tissue and animal source of mesenchymal stromal cells (MSCs) were extracted from 206 included articles. As well, the immunocompatibility of extracellular vesicles (EVs) were determined by comparing the animal source of MSCs to animal model of disease. Articles may have utilized EVs from more than one animal and/or tissue source.

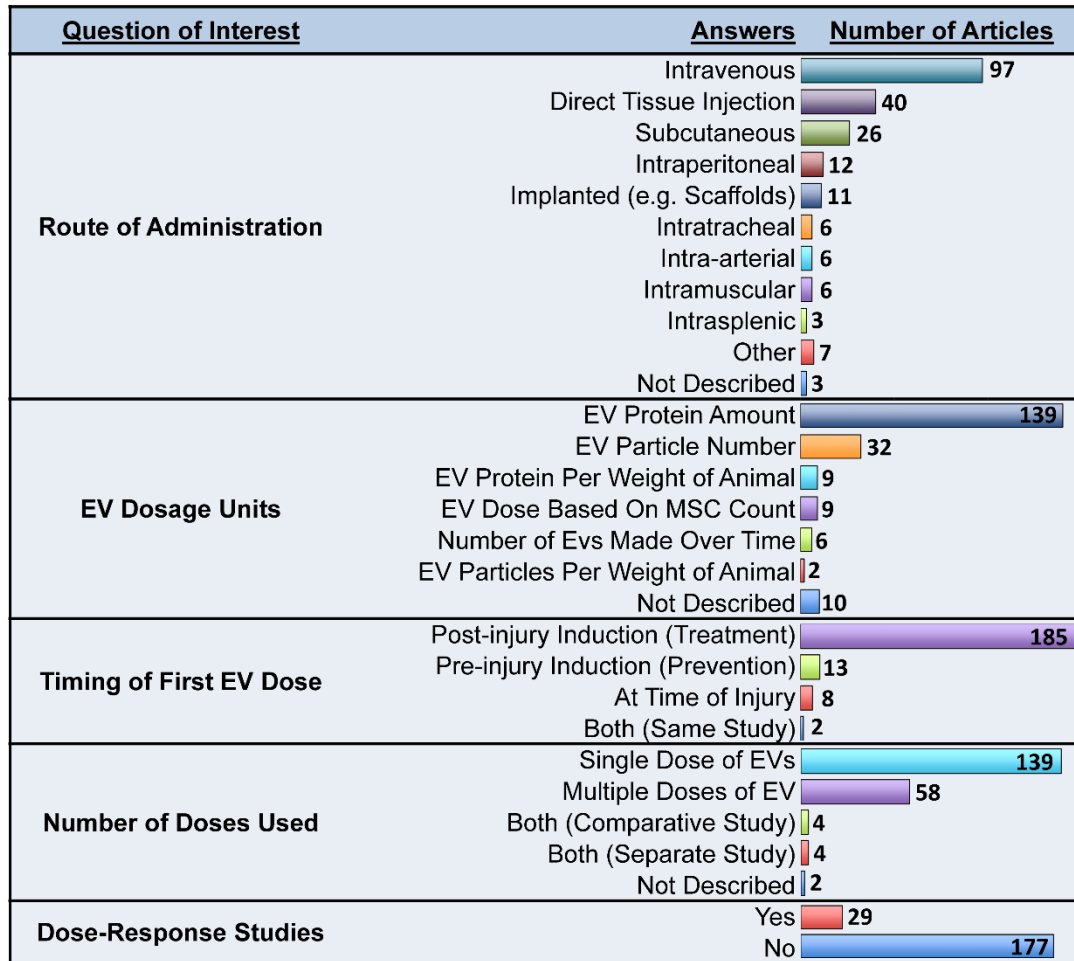


Figure 3.7. Overview dosage regimen of administered MSC-EVs.

Details pertaining to route of administration, units of dose used, number of doses, timing of administration and prevalence of dose-response studies was extracted from 206 articles. Studies may have may have incorporated more than one route of administration.

Question of Interest	Answers	Number of Articles
Modification of EVs	Unmodified	176
	Modified MSCs Before EV Isolation	83
	Modified EVs Directly	37
	Co-injected with Another Treatment	23
MSC Transfection or Transduction (N=49)	siRNA or shRNA Targeting Proteins	27
	microRNA Targets	20
	Protein Mutations	2
EV Preconditioning (N=36)	RNA Enzymatic Digestion	13
	Scaffold Implantation	12
	Protein Enzymatic Digestion	5
	Incubation with Antibodies or shRNA	5
	Hyaluronic Acid	1
MSC Preconditioning (N=29)	Hypoxia	12
	Pro-Inflammatory Cytokine	5
	Lipopolysaccharide	3
	Polyinosinic:Polycytidylic Acid (Poly I:C)	2
	Co-Culturing with Cells	2
	Others	5

Figure 3.8. Overview of studies that incorporated modifications to MSC-EVs.

Type of modiciations were subcategorized by MSC transfections/transductions, preconditioning of EVs before administration, or preconditioning of MSCs before EV isolation.

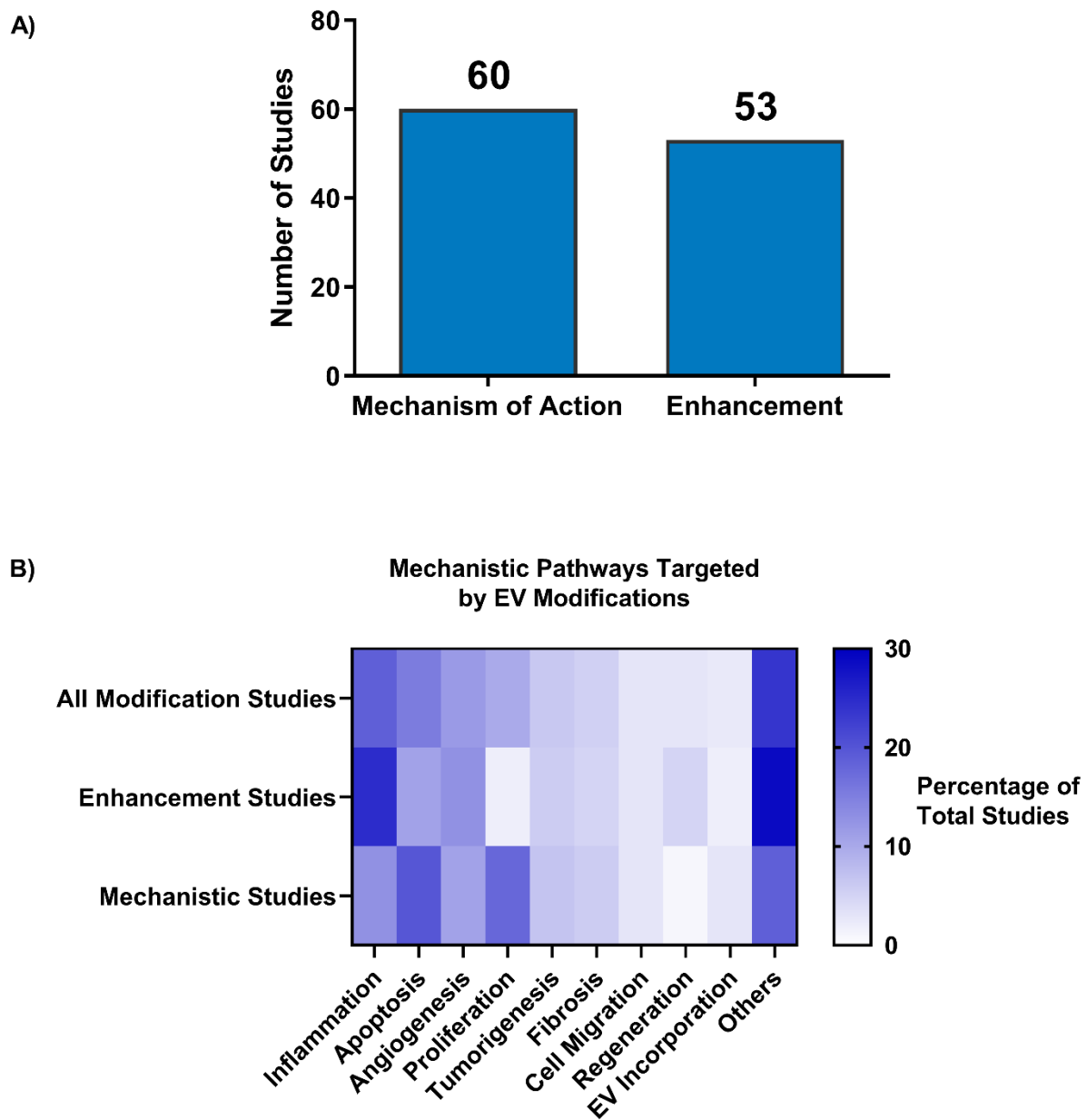


Figure 3.9. Analysis of preclinical animal studies involving modified MSC-EVs

(A) Modification studies that explored mechanism of action (N=60) compared to enhancements (N=53). (B) Heat map displaying the most targeted pathways by studies of EV modifications. Studies of enhancement more commonly focused on immunomodulation, whereas mechanistic studies favored the anti-apoptotic and pro-proliferative effects of MSC-derived EVs.

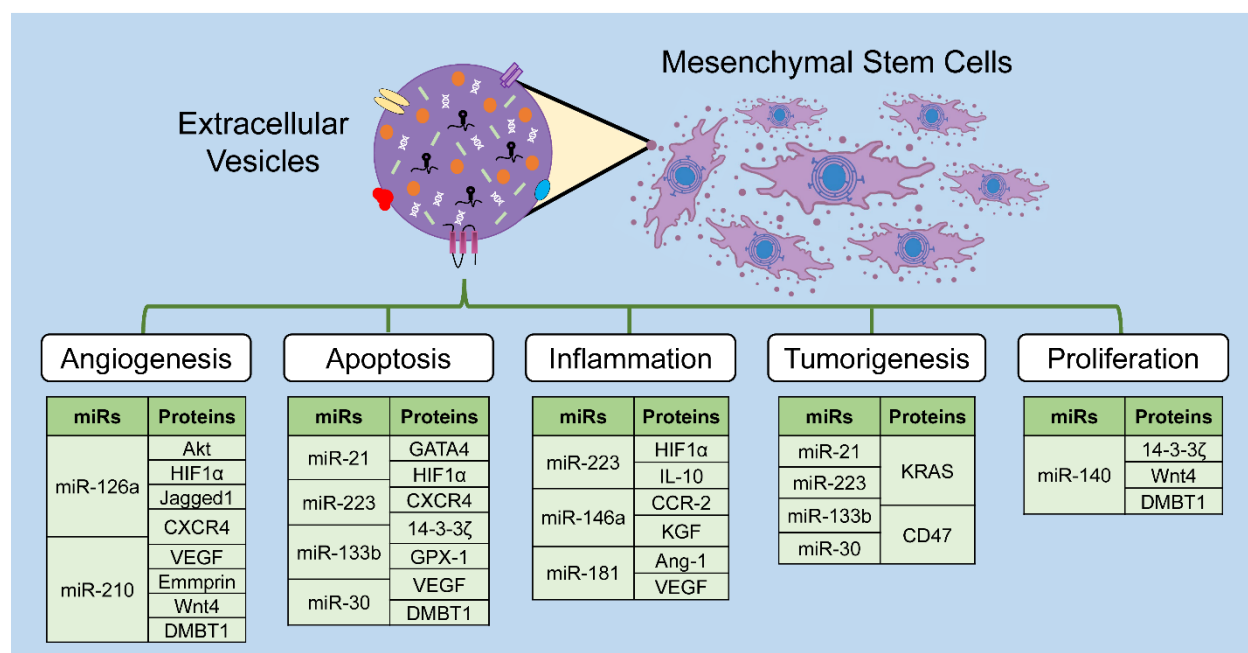


Figure 3.10. Overview of the microRNA and proteins that were demonstrated to be critical for MSC-EV efficacy.

Analysis of 113 animal studies investigating MSC-EV modifications. The most commonly targeted cellular pathways by EVs were angiogenesis, apoptosis, inflammation, tumorigenesis and proliferation. miR = microRNA; Akt = protein kinase B; HIF = hypoxia inducible factor; CXCR4 = C-X-C chemokine receptor type 4; VEGF = vascular endothelial growth factor; DMBT1 = deleted in malignant brain tumor 1 protein; GPX1 = glutathione peroxidase 1; IL-10 = interleukin 10; CCR-2 = C-C chemokine receptor type 2; KGF = keratinocyte growth factor; Ang-1 = angiopoietin 1.

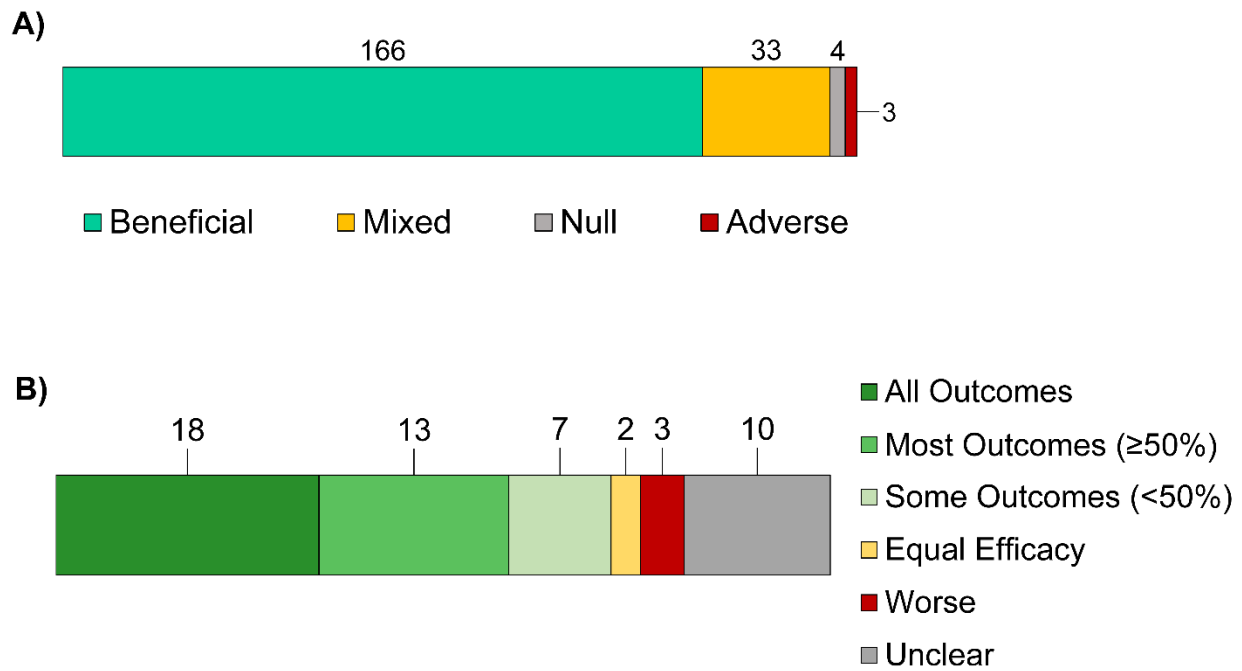


Figure 3.11. Analysis of outcomes from MSC-EV studies.

(A) Overview on general outcomes from 206 included articles. Studies were categorized as “beneficial” if extracellular vesicles (EVs) improved all reported outcomes, “mixed” if at least one of the outcomes were not improved by EVs, “null” if no benefits were observed, or “adverse” if administration of EVs resulted in worse outcomes. (B) Outcomes comparing modified (enhanced) EVs to unmodified EVs in N=53 animal studies.

Chapter 4 – Mesenchymal Stromal Cell Extracellular Vesicles as Therapy for Acute and Chronic Respiratory Diseases: A Meta- Analysis

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Author Contributions:

AT: Created the systematic review protocol, conducted the literature search, assessed study eligibility and completed data extraction. Contributed to data analysis, wrote the manuscript, generated the tables/figures and addressed reviewer comments/revisions.

MML, KH and CG: Contributed to writing of manuscript, creation of protocol, and finalizing methodological approach. Assisted in addressing reviewer comments/revisions and generating tables/figures.

JM and DAF: Contributed in data analysis and review of manuscript.

DSA, DJS and BT: Assisted in developing systematic review protocol and review of manuscript.

4.1 Abstract

Preclinical studies suggest mesenchymal stromal cell extracellular vesicles (MSC-EVs) reduce inflammation and improve organ function in lung diseases; however, an objective analysis of all available data is needed prior to translation. Using rigorous meta-research methods, we determined the effectiveness of MSC-EVs for preclinical respiratory diseases and identified experimental conditions that may further refine this therapy. A systematic search of MEDLINE/Embase identified 1167 records. After screening, 52 articles were included for data extraction and evaluated for risk of bias and quality of reporting in study design. A random effects meta-analysis was conducted for acute lung injury (ALI; N=23), bronchopulmonary dysplasia (BPD; N=8) and pulmonary arterial hypertension (PAH; N=7). Subgroup analyses identified EV methods/characteristics that may be associated with improved efficacy. Data is presented as standardized mean differences (SMD) or risk ratios (RR) with 95% confidence intervals (CI). For ALI, MSC-EVs markedly reduced lung injury (SMD 4.33, CI 2.92-5.73), vascular permeability (SMD 2.43, CI 1.82-3.05), and mortality (RR 0.39, CI 0.22-0.68). Small EVs were more consistently effective than large EVs whereas no differences were observed between tissue sources, immunocompatibility or isolation techniques. For BPD, alveolarization was improved by MSC-EVs (SMD 1.45, CI 0.82-2.08) with small EVs more consistently beneficial than small/large EVs. In PAH, right ventricular systolic pressure (SMD 4.16, CI 2.64-5.68) and hypertrophy (SMD 2.80, CI 1.91-3.68) were significantly attenuated by EVs. In BPD and PAH, EVs isolated by ultracentrifugation demonstrated therapeutic benefit whereas tangential flow filtration (N=2) displayed minimal efficacy. Lastly, risk of bias and quality of reporting for experimental design were consistently unclear across all studies. Our findings demonstrate clear potential of MSC-EVs to be developed as therapy for acute and chronic lung diseases. However, greater transparency in

research design and direct comparisons of isolation technique and EV subtypes are needed to generate robust evidence to guide clinical translation.

Protocol Registration: PROSPERO [CRD42020145334](https://www.crd42020145334)

Keywords: Systematic Review, Mesenchymal Stem Cells, Mesenchymal Stromal Cells
Extracellular Vesicles, Exosomes, Respiratory, Lung, Meta-Analysis

4.2 Introduction

Mesenchymal stromal cells (MSCs) are a heterogeneous population of cells with multipotent differentiation potential and diverse secretory, immunomodulatory, and therapeutic functions.^{175,360} They can be derived from readily available tissues, such as bone marrow, umbilical cord, and adipose tissue.^{361,362} Many *in vitro* and *in vivo* studies show that MSCs can modulate inflammatory pathways, improve organ function and prolong survival,^{361,363} and there are currently over 1000 registered clinical trials registered on ClinicalTrials.gov investigating MSCs as an intervention across a variety of diseases. Despite this widespread interest, MSCs have received approval for only two conditions, graft versus host disease and perianal fistulas in Crohn's disease.¹⁹⁶ The lack of successful clinical translation is multifactorial but may include technical challenges associated with generating a consistent, viable and effective cell therapy.³⁶⁴⁻³⁶⁶ For example, post-hoc analysis from a clinical trial of MSCs for moderate to severe acute respiratory distress syndrome demonstrated an extensive range in cell viability from 36 to 85%.²⁰⁷ Interestingly, it has now been suggested that MSCs exert their therapeutic effects at least in part by secretion of extracellular vesicles (EVs), which may represent a more robust therapeutic product than delivery of intact cells.²¹³⁻²¹⁷

Respiratory disorders, whether acute or chronic, remain a leading cause of death worldwide.³⁶⁷ Greater than 500 million people are affected by chronic lung diseases with minimal curative options.³⁶⁸ Moreover, the sudden onset of COVID-19 related acute respiratory distress syndrome has shed light on the lack of available specific therapies to combat severe respiratory infections/injury. Given the anti-inflammatory and regenerative properties of MSC-derived EVs (MSC-EVs), pulmonary diseases have become a prominent focus for preclinical development of EV therapeutics.³⁶⁹ Administration of MSC-EVs improved outcomes in both acute (e.g. acute lung

injury, the preclinical correlate of acute respiratory distress syndrome) and chronic (e.g. pulmonary arterial hypertension) conditions, as well as neonatal diseases (e.g. bronchopulmonary dysplasia).^{265,370-372} MSC-EVs carry a variety of key mRNAs, microRNAs and proteins known to mediate cell processes including inflammation, angiogenesis, apoptosis and fibrosis,²⁴⁷ all of which are critical to lung recovery. Hence, EVs can potentially harness the benefits of whole cells while overcoming many of the common issues that accompany live cell therapies. These advantages include EVs potentially having lower immunogenicity, ability to cross important biological barriers (e.g. lung endothelium and epithelium), and favorable manufacturing/storage procedures.³⁴⁰

As EV therapies have garnered interest over the past decade, there remain challenges and unanswered questions associated with developing this cell-free therapy. In our previous systematic review, we analyzed the methodology and outcomes of more than 200 *in vivo* studies investigating MSC-EVs as a therapy.²⁴⁷ There was high heterogeneity in EV nomenclature, experimental approach, interventional traits, dosage regimen and study design. The Minimal Information for Studies of Extracellular Vesicles (MISEV 2018) attempts to address many of these issues.²²⁷ However, given the diversity of EV methodology available, there is no consensus as to which isolation technique, interventional traits (e.g. MSC source or EV subtype) or administration protocols (e.g. delivery route, timing of treatment) will result in greatest therapeutic benefit. A systematic and comprehensive understanding of these experimental details and the overall impact of MSC-EVs on lung repair are needed prior to considering clinical translation.

In the clinical world, scientifically rigorous systematic reviews and meta-analyses are the gold standard approach for evaluating the efficacy of interventions objectively and comprehensively.^{283,373-375} They objectively assess the quality of research being conducted,

elucidate specific therapy or patient population characteristics that affect treatment outcomes, and identify knowledge gaps. By applying meta-research methods in preclinical EV research, our study aims to determine the efficacy of MSC-EVs as treatment for various acute and chronic respiratory conditions. A meta-analysis of acute lung injury, bronchopulmonary dysplasia, and pulmonary arterial hypertension was completed. We also conducted subgroup analyses to explore the specific EV characteristics and methodology that may be associated with greater benefits to help refine MSC-EV therapy. Lastly, we assessed the quality of reporting experimental parameters and risk of bias in study design that currently exists in MSC-EV research.

4.3 Materials and Methods

4.3.1 Protocol and Registration

Our protocol was registered in the International Prospective Registry of Systematic Reviews (PROSPERO [CRD42020145334](https://www.crd42020145334)). Reporting of this systematic review follows the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 guidelines (Supplemental File C1).

4.3.2 Eligibility Criteria

Eligibility criteria for study inclusion was created *a priori* and defined according to five parameters: population, intervention, comparator, outcomes and study design (PICOS). Predefining PICOS parameters is standard practice in meta-research methodology that enables unbiased search, inclusion, and assessment of pertinent articles.

Population: We included any *in vivo* animal models of respiratory disease or injury. Invertebrate animals, *in vitro* or *ex vivo* preclinical studies were excluded.

Intervention: Studies must have administered MSC-EVs for inclusion. Any animal or tissue source of MSCs were included. MSCs or EVs may have been pre-treated, co-treated or modified (e.g. gene transfection). Any route, dose, timing or frequency of administration was included. Studies may have administered EVs as a xenogeneic, allogeneic or autologous protocol. Studies in which EVs were not derived from MSCs or were not administered to animals directly was excluded.

Comparator: Any comparator including (but not limited to) placebo, vehicle control, MSCs and/or fibroblasts was considered.

Outcome: Primary, secondary and tertiary outcomes were selected for acute lung injury (ALI), bronchopulmonary dysplasia (BPD), and pulmonary arterial hypertension (PAH), the three most studied lung conditions for MSC-EV therapy. For lung conditions outside of ALI, BPD, and PAH, the primary and secondary outcomes reported in the articles were extracted for narrative synthesis. Studies in which outcomes did not assess EV efficacy (e.g. only the biodistribution of EVs was assessed) were excluded.

Study Design: We included controlled interventional studies (randomized, pseudo-randomized, or non-randomized), while unpublished gray literature, abstracts, review articles, editorials, commentaries, and letters were excluded. Studies were not excluded based on language or publication date.

4.3.3 Search Strategy

A systematic search of Ovid MEDLINE®, Ovid MEDLINE® In- Process & Other Non-Indexed Citations, and Embase Classic + Embase was conducted until March 6, 2019 using a broad search strategy for any *in vivo* study investigating MSC-EVs. To update our study, an additional search was conducted on August 4, 2020 with lung disease filters. Both search strategies

(**Supplemental File C2**) were designed with the help of an information specialist and assessed by a second information specialist through the Peer Review of Electronic Search Strategies (PRESS).⁷³ The search strategies were modified according to database and used controlled vocabulary, MeSH terms (e.g. mesenchymal stem cells, mesenchymal stromal cells, extracellular vesicles, exosomes, microvesicles), abbreviations (e.g. MSCs, EVs, MVs), and filters for preclinical animal models. No restrictions were applied for publication date.

4.3.4 Study Selection and Data Extraction

Titles and abstracts were independently screened in duplicate (AT, MS, CG and/or KH) using the Distiller Systematic Review Software (DistillerSR, Evidence Partners, Ottawa, Canada). A calibration test using 10 studies was performed before beginning the formal screening process to ensure high inter-rater correlation (kappa 0.8). Titles and abstracts that met criteria or that could not be excluded with certainty moved on to full-text screening. Following a calibration test involving 10 studies, two reviewers (AT, MS, CG and/or KH) independently reviewed the full-text articles. Reasons for exclusion were recorded at this stage. Disagreements between reviewers were resolved through discussion. The selection process is presented in a PRISMA flow diagram (**Figure 4.1**).

Standardized data extraction forms were created in DistillerSR. Data pertaining to study characteristics, risk of bias, and quality assessment were extracted independently and in duplicate (AT, CG and/or KH). A calibration exercise for data extraction was conducted on the first three studies. Data was collected on study characteristics, including: general study characteristics, animal model characteristics (e.g. species, sex, model of disease or injury), MSC and EV isolation and characterization techniques, MSC or EV modifications, and dosage. Details for all data collected can be found in our registered PROSPERO protocol (**Supplemental Files C6 and C7**).

We used the generally accepted size-based definitions of EV subtypes, *i.e.* exosomes or small EVs (30-150nm in diameter) and microvesicles or large EVs (150-1000nm in diameter),^{227,247} to determine whether authors used these terms consistent with MISEV recommendations.

Since MSCs are a heterogeneous population of cells widely accessible from a variety of tissue sources, the International Society for Cell Therapy (ISCT) published international guidelines to standardize MSC characterization. Studies were assessed for their adherence to the ISCT criteria including: (1) adherence to plastic in standard culture conditions, (2) positive and negative expression of specific surface antigens, and (3) multipotent differentiation potential. Furthermore, international guidelines for investigating EVs were published in 2018 entitled “Minimal Information for Studies of EVs” (MISEV 2018).²²⁷ Researchers are encouraged to characterize EVs by amount, two measures of single vesicle analysis, and at least three positive and one negative protein marker. These criteria were used to assess study adherence to EV characterization.

4.3.5 Primary and Secondary Outcomes

Outcomes were predetermined by consulting senior scientists who have had extensive experience within the fields of regenerative medicine and lung disease. Primary outcomes included histological lung injury scores for ALI, mean linear intercept (a measure of alveolarization) for BPD, and right ventricular systolic pressure for PAH. Secondary outcomes for ALI and BPD included protein concentration and neutrophil count in bronchoalveolar lavage fluid (measures of alveolar-capillary membrane permeability and inflammation, respectively). For BPD and PAH, Fulton's index (a measure of right ventricular hypertrophy and indicator of pulmonary hypertension) was extracted as a secondary outcome. Tertiary outcomes, if reported, included measures of survival and adverse events of EV therapy. The corresponding authors of the individual studies included in our review were contacted to obtain relevant missing data.

4.3.6 Risk of Bias and Quality of Reporting

Risk of bias was evaluated by two independent reviewers (AT, CG) using the SYRCLE (Systematic Review Centre for Laboratory Animal Experimentation) risk of bias tool.³¹⁰ The SYRCLE tool features 10 different parameters including randomization, blinding and outcome reporting, each of which was scored as having a low, high, or unclear risk of bias for each study. Quality of reporting was evaluated by two independent reviewers (AT, CG) using the NIH standards for transparency in reporting preclinical research. Each element in the NIH standards for transparency was scored as having been reported or not reported. Disagreements between reviewers were resolved through discussion.^{376,377}

4.3.7 Data Analysis

Extracted data on study characteristics, methodology and intervention characteristics are presented as frequencies, proportions and/or percentages. Studies may have used multiple techniques and interventions, either as separate experiments or in combination. Hence, the cumulative percentage of certain categories may be greater than 100%.

Study outcomes were pooled using Comprehensive Meta-Analyst (version 3; BioStat Inc., USA) and represented by forest plots. Continuous outcome measures were expressed as standardized mean differences (SMDs) with 95% confidence intervals (CIs), which were calculated using a random effects inverse variance meta-analysis. Standardized mean difference was chosen due to the expected heterogeneity in the measurement techniques of outcomes. Dichotomous outcome measures were expressed as risk ratios (RRs) with 95% confidence intervals (CIs), which were calculated using a random effects meta-analysis based on the DerSimonian Laird model. We corrected for the multiple use of a single control group using the method described by Vesterinen *et al.*³⁷⁸ Statistical heterogeneity was assessed using the Cochrane

I² test. Planned subgroup analyses included subgroups based on animal characteristics (e.g. sex, age, species), intervention characteristics (e.g. tissue source of MSCs, subtype of EVs, method of EV isolation), timing of treatment and respiratory disease modelled (e.g. ALI, BPD, PAH).

4.4 Results

4.4.1 Study Characteristics

Our systematic searches yielded a total of 1167 records (**Figure 4.1**). Following level 1 screening of titles and abstracts, 434 articles underwent level 2 full-text screening which resulted in a total of 52 studies included for our systematic review.^{269,379-429} A meta-analysis focussed on the three most common disease categories (ALI, BPD and PAH).

Baseline characteristics of the included studies are reported in **Table 4.1**. A majority of papers were published from the United States (N=22; 42%) or China (N=21; 42%) (**Figure S3.1**). The first paper examining MSC-EVs in a model of lung disease was published in 2012. Since then, there has been a marked growth in the number of preclinical animal studies investigating MSC-EVs with over 60% of studies (N=32) published in 2019 and 2020 (**Figure S3.1**). A variety of lung conditions have been investigated including ALI (N=23; 44%), PAH (N=8; 15%), BPD (N=7; 13%), asthma / allergic inflammation (N=5; 10%), pulmonary fibrosis (N=5; 10%), congenital diaphragmatic hernia (N=2; 4%) and chronic obstructive pulmonary disease (N=2; 4%). Most studies utilized either a mouse (N=31; 60%) and/or rat (N=21; 40%) model, with only one study applying a pig model (2%). Nearly half of the studies used only male animals (N=24; 46%), with 8 studies using females only (15%), 2 studies using both sexes (4%), and 18 studies not reporting of the sex of their animals (35%).

4.4.2 EV Methodology and Interventional Characteristics

The animal source of MSCs used included human (N=34; 65%), mouse (N=11; 21%), rat (N=8; 15%) and pig (N=1; 2%) (**Figure S3.2**). MSCs were derived from a variety of tissue sources including bone marrow (N=31; 60%), umbilical cord (N=11; 21%), adipose tissue (N=6; 12%), placenta (N=3; 6%) and induced pluripotent stem cells (iPSCs) (N=2; 4%) (**Figure S3.2**). A majority of studies applied a xenogeneic administration (N=34; 65%) as opposed to allogeneic (N=20; 38%). Based on the ISCT guidelines, 30 studies (58%) met all three criteria for MSC characterization. *In vitro* trilineage differentiation and the positive/negative markers used in each study are described in **Figure S3.2**.

The most common techniques used for EV isolation were ultracentrifugation (N=34; 65%), isolation kits (N=7; 13%) and tangential flow filtration (N=6; 12%) (**Figure S3.3**). EVs were characterized by quantification (N=47; 90%), size distribution (N=45; 87%), morphological analysis (N=45; 87%) and/or surface marker expression (N=41; 79%) in most studies (**Figure S3.3**). Based on the MISEV 2018 guidelines, 10 studies (19%) adequately characterized their EV therapies. Importantly, 52% (N=27) of studies did not report assessment of negative markers to demonstrate specific isolation of EVs. Regarding EV nomenclature, we assessed whether the terminology used was consistent with reported size distribution of EVs (e.g. small, large or both small/large EVs combined). Thirty-five (67%) studies utilized consistent terminology, whereas 8 studies (15%) used discordant terms and 9 studies (17%) did not provide size analysis. More details regarding EV isolation and characterization, including specific techniques and markers used, can be found in **Figure S3.3**.

The two most common routes of administration for MSC-EV delivery included intravenous (N=31; 60%) and intratracheal (N=16; 31%) delivery (**Figure S3.4**). The units applied for dosing EVs varied considerably and included absolute protein amount (N=23; 44%), particle number

(N=11; 21%) or amount of EVs released by a certain number of MSCs (N=8; 15%), as well as EVs released over time or dosed by weight of animal (**Figure S3.4**). Most studies delivered a single dose of therapy (N=35; 67%); studies with multiple administrations used a median value of 4 doses (ranging from 2-15 doses). EV modifications were conducted in 33 studies (63%) which included modifications before EV isolation (N=17; 33%), modifications to EVs directly (N=8; 15%) and co-treatments (N=8; 15%). Lastly, dose-response and biodistribution analyses were examined in 8 (15%) and 7 (13%) studies, respectively (**Figure S3.4**).

4.4.3 Meta-Analyses of Acute Lung Injury Studies

Method of ALI induction included intratracheal endotoxin (e.g. lipopolysaccharide; N=8), intratracheal *E. Coli* (N=3), ischemia-reperfusion injury (N=2), bleomycin injection (N=2), smoke inhalation (N=1), radiation (N=1), viral induction (N=1), shock (N=1), phosgene (N=1), burn (N=1), trauma (N=1) or intestinal ischemia-reperfusion (N=1). Histological lung injury was reported in 7 studies investigating preclinical ALI. All 7 studies demonstrated a significant reduction in lung injury from MSC-EV therapy (pooled analysis, SMD -4.33, 95%CI -5.73 to -2.92, $I^2 = 74\%$) (**Figure 4.2**). Subgroup analyses demonstrated that small EVs consistently result in a significant attenuation to lung injury whereas the efficacy of large EVs is highly variable and similar to control treatments from pooled analysis (**Figure 4.3**). Studies that isolated EVs by ultracentrifugation did not demonstrate a difference in effect size as compared to the single study that utilized an isolation kit (**Figure 4.3**). Lastly, MSC-EVs may demonstrate greater benefits ($P=0.004$) to attenuate lung injury in female rodents as compared to males, however only one study used female animals (**Figure 4.3**). No difference in effect size was observed between MSC tissue source (bone marrow vs. umbilical cord), species source of MSCs, immunocompatibility

(allogeneic vs. xenogeneic), timing of treatment, route of administration or animal model (**Figure 4.3**).

Changes to alveolar-capillary barrier permeability measured by bronchoalveolar lavage fluid (BALF) protein concentration was reported in 28 studies. Overall, MSC-EV administration is associated with a significant attenuation to lung permeability (SMD -2.43, 95%CI -3.05 to -1.82, $I^2 = 83\%$) (**Figure 4.4**). From subgroup analysis, lung permeability was reduced to a greater extent ($P < 0.001$) by delivery of small EVs or both small/large as compared to large EVs alone (**Figure 4.5**). Allogeneic administration of MSC-EVs also showed a more significant reduction to lung permeability as compared to xenogeneic delivery ($P = 0.001$). Sex ($P = 0.009$) and species ($P = 0.03$) may be associated with greater EV efficacy as female animals and rat models demonstrated better outcomes (**Figure 4.5**). Lastly, the single study that used an isolation kit displayed a greater improvement ($P < 0.001$) to lung permeability as compared to studies that utilized ultracentrifugation. Similar to lung injury score, no difference in effect size was observed when considering MSC tissue source, route of administration or timing of treatment.

Other secondary outcomes considered for ALI studies included mortality (RR 0.39, 95%CI 0.22 to 0.68, $I^2 = 14\%$) (**Figure S4.1**) and inflammatory response as measured by neutrophil infiltration in BALF (SMD -1.42, 95%CI -1.83 to -1.02, $I^2 = 66\%$) (**Figure S4.2**), both of which were significantly improved by MSC-EV therapy. Lastly, a dose-response analysis was conducted for studies administering EVs by protein amount and reporting lung vascular permeability as an outcome. Despite many studies delivering similar doses, the effect size varied considerably and was not correlated to dose ($P = 0.311$) (**Figure S4.3**).

4.4.4 Meta-Analyses of Bronchopulmonary Dysplasia Studies

All animal models of BPD were induced by housing newborn mice or rats under hyperoxia conditions, ranging from 60-95% oxygen. Alveolarization was reported in 11 studies from 7 different articles and pooled analysis demonstrated marked improvements from MSC-EV administration (SMD -1.45, 95%CI -2.08 to -0.82, $I^2 = 63\%$) (**Figure 4.6**). Similar to ALI, subgroup analysis indicated that small EVs consistently improve alveolarization, whereas the combination of small/large EVs were highly variable and not statistically different as compared to control (**Figure 4.7**). One study that isolated EVs by TFF did not exhibit any benefits from EVs. However, when TFF is combined with density gradient ultracentrifugation or ultracentrifugation is used alone, MSC-EVs significantly improved lung alveolarization ($P=0.008$) (**Figure 4.7**). Immunocompatibility (allogeneic, $P=0.045$), route of administration (intraperitoneal, $P=0.040$) and timing of treatment (concurrent, $P=0.044$) may result in differential effects, whereas no differences in efficacy was observed when comparing tissue source of MSCs or animal species.

One study evaluated the survival benefits of MSC-EVs in experimental BPD; however, mortality was shown to be unaltered as compared to control (RR 0.86, 95%CI 0.41 to 1.80, $I^2 = 0\%$) (**Figure S4.1**). Other secondary outcomes of experimental BPD that were assessed include alveolar permeability (SMD -1.80, 95%CI -3.18 to -0.41, $I^2 = 67\%$) (**Figure 4.4**) and lung inflammation as measured by BALF neutrophil (SMD -2.48, 95%CI -3.41 to -1.75, $I^2 = 66\%$) (**Figure S4.2**), both of which demonstrated significant improvements from MSC-EV delivery. Furthermore, pulmonary hypertension is a common complication of BPD. MSC-EV delivery resulted in marked reductions to both Fulton's Index (measure of right ventricular hypertrophy; SMD -0.99, 95%CI -1.49 to -0.49, $I^2 = 0\%$) (**Figure S4.4**) and RVSP (SMD -1.20, 95%CI -2.07 to -0.34, $I^2 = 0\%$) (**Figure S4.5**).

4.4.5 Meta-Analyses of Pulmonary Arterial Hypertension Studies

Method for PAH induction included monocrotaline injection (N=5), chronic hypoxia(N=2), or the combination of semaxanib (SU5416, a VEGF inhibitor) and chronic hypoxia in rats (N=2). Ten distinct studies from 5 articles reported RVSP, each of which demonstrated a significant reduction after MSC-EV administration (pooled analysis, SMD -4.16, 95%CI -5.68 to -2.64, $I^2 = 84\%$) (**Figure 4.8**). Exploratory subgroup analysis showed allogeneic MSC-EVs improved RVSP to a significantly greater extent than xenogeneic administration ($P<0.001$) (**Figure 4.9**). Interestingly, in the single report of which EVs were isolated by combining TFF and size exclusion chromatography, EVs were less efficacious ($P<0.001$) as compared to ultracentrifugation or ultracentrifugation/chromatography combined (**Figure 4.9**). Studies that did not report sex showed a significantly greater reduction in RVSP as compared to male-only studies ($P<0.001$). Despite the well-known sex dependency of PAH, no preclinical study explored MSC-EV therapy in female animals. Unlike ALI or BPD, EV subtype did not demonstrate variable effects to RVSP. No difference in EV efficacy were observed when comparing MSC tissue source or timing of treatment (**Figure 4.9**). Lastly, 17 studies reported the secondary outcome of Fulton's Index of which MSC-EV injection led to a significant reduction in right ventricular hypertrophy (SMD -2.80, 95%CI -3.68 to -1.91, $I^2 = 78\%$) (**Figure S4.4**).

4.4.6 Studies of Other Respiratory Conditions

Asthma / Allergic Airway Inflammation (N=5 studies)

Disease was induced by intraperitoneal delivery of ovalbumin (N=3),⁴³⁰⁻⁴³² *Aspergillus fumigatus* hyphal extract (N=1),⁴³³ or IL-33 (N=1)⁴³⁴. MSC-EVs were administered by intravenous injection in 4 studies, with one study using intranasal delivery for more localized lung distribution. Four studies assessed *in vivo* outcomes, all of which reported a significant reduction to BALF neutrophils, eosinophils and pro-inflammatory cytokines from MSC-EV delivery. Three studies

reported a marked attenuation in lung inflammation score. One study showed improvements to lung mechanics, and another demonstrated a reduction in lung hyperresponsiveness to allergens (e.g. *Aspergillus* hyphal). Lastly, one study assessed differential gene expression in lung tissues post-EV administration and found the most upregulated genes were those related to antioxidation (*Pon1*), mitochondrial function (*Bex2*) and lung epithelium protection (*Scgb1c1*). Of note, the inhibition of miR-146a abolished the therapeutic efficacy of MSC-EVs, thereby illustrating its critical role within EVs for immunomodulation.

Pulmonary Fibrosis (N=5 studies)

Fibrosis was induced by intratracheal silica (N=2),^{435,436} bleomycin (N=2),^{437,438} or fine particulate matter (N=1)⁴³⁹. All studies delivered MSC-EVs as a rescue treatment which led to reduced lung collagen content in 3 studies, one of which was still significantly elevated as compared to control. In the other 2 studies, MSC-EV treated animals did not attenuate collagen deposition, whereas MSCs themselves or lung spheroid cell exosomes led to marked improvements. The effect of MSC-EVs on BALF macrophage expression was variable. Two studies reported no changes in number of lung macrophages, whereas one study observed a rescued expression of anti-inflammatory macrophages. Most studies demonstrated reduced expression of pro-inflammatory cytokines including IL-1 β , TGF β 1 and TNF α . Lastly, one study found that transplantation of bone marrow derived monocytes preconditioned with MSC-EVs attenuated both pulmonary fibrosis and lung inflammation.

Chronic Obstructive Pulmonary Disease (COPD)

Two articles (4%) studied the effects of MSC-EVs in a cigarette smoke induced mouse model of COPD.^{440,441} In one study, the combination of MSCs and exosomes were more effective than either treatment alone to reduce BALF cell count and cytokine levels.⁴⁴¹ The mechanism of

action was found to be recovery of mitochondrial transfer and mitochondrial fusion gene expression. The second study of COPD found intraperitoneal delivery of MSC-EVs resulted in marked reductions to plasma pro-inflammatory cytokines, histological lung injury and lung influx of inflammatory immune cells.⁴⁴⁰ Authors tested their EV product in a clinical study of 30 patients. In all subjects, inhalation of MSC-EVs significantly enhanced pulmonary function as measured by forced expiratory volume in 1 second (FEV1), peak expiratory flow rate, and the 6-minute walking test. Computed tomography scans showed a reduction in emphysematous changes and decreased lung hyper-expansion. EVs were well-tolerated with improvements to quality of life and no adverse events.

4.4.7 Quality of Reporting in MSC-EV Research

All 52 lung articles were assessed for their completeness of reporting study design domains based on the NIH Principles and Guidelines for Reporting in Preclinical Research.³⁷⁶ These guidelines identify parameters that should be included in preclinical publication to enhance scientific rigor, reproducibility and transparency. No articles utilized established reporting guidelines (e.g. Animal Research: Reporting *In Vivo* Experiments, ARRIVE) (**Figure 4.10**). Findings were substantiated under a range of conditions (e.g. different dosages, routes of administration, modifications) in 31 studies (60%). Forty studies (77%) stated N-values for at least one *in vivo* outcome and 31 studies (60%) for all outcomes. *A priori* power calculations of sample size were described in 7 studies (13%); however, all 7 studies had inadequate information necessary to calculate sample size. The total number of animals used in all experiments were reported in 17 studies (33%) (**Figure 4.10**).

A primary outcome was not explicitly stated in any of the 52 studies (**Figure 4.10**). The number of measurements per subject was described for at least one outcome in 30 studies (58%),

2 of which reported the number of replicates in all findings. Randomization of experimental groups was mentioned in less than half of the articles (N=24); however, details for method of randomization was not described in any article. No studies reported blinding of the personnel conducting experiments. Blinding of data analysis was described for all outcomes in 2 studies (4%) and some outcomes in 16 studies (31%), with no blinding of outcome assessment mentioned for 33 studies (66%). Lastly, the NIH recommends reporting the criteria for data exclusion to minimize selection bias. Seven studies (13%) provided reasoning for data omission which included excluding animals with heart rates <300 beats per minute for hemodynamic measurements,²⁶⁰ low expression of genes in RNA sequencing,²⁶⁴ or statistical outliers⁴⁴². Forty-five studies (87%) did not provide any criteria for data exclusion (**Figure 4.10**).

4.4.8 Risk of Bias Assessment

A majority of studies were deemed as having an “unclear” risk of bias across most domains (**Figure 4.11**). Although 24 studies (46%) reported that animals were randomized to experimental groups, no studies provided details regarding method of *randomization* (i.e. to reduce selection bias) which is essential to assess adequate sequence generation. Thirty-two studies (62%) displayed a low risk for reporting *baseline characteristics* (i.e. to reduce selection bias). However, the risk of bias was unclear across all studies for the domains of *allocation concealment* (i.e. selection bias), *random housing* (i.e. selection bias), *blinding of personnel* (i.e. performance bias) and *selective outcome reporting* (i.e. reporting bias). Seventeen studies reported *blinded assessments* (i.e. detection bias) for some outcomes (e.g. histological/pathological injury) and 2 studies were blinded for all outcomes. *Incomplete outcome data* (i.e. attrition bias) was assessed as whether the N-value was consistent between the methods and results sections. Fourteen studies (27%) exhibited low risk, 25 studies (48%) had unclear risk and 13 studies (25%) were deemed

high risk. Lastly, *other potential sources of bias* considered included source of funding, sample size calculation and conflict of interests. Eleven studies (21%) had a high risk in at least one of these additional categories. (**Figure 4.11**).

4.5 Discussion

Our systematic review provides a comprehensive synthesis of the preclinical efficacy, methodology, study design and preclinical reporting of MSC-EV studies of acute and chronic respiratory diseases. Pooled estimates from meta-analyses indicate that MSC-EV administration significantly improves primary outcomes in ALI (i.e. lung injury score), BPD (i.e. alveolarization) and PAH (i.e. right ventricular systolic pressure). With the exception of mortality for BPD, MSC-EVs were also markedly effective at improving secondary outcomes including survival (ALI), lung vascular permeability (ALI and BPD), BALF neutrophil count (ALI and BPD), RVSP (BPD) and Fulton's Index (BPD and PAH). These findings demonstrate the therapeutic efficacy of MSC-EVs to treat both acute and chronic lung diseases.

Our previous systematic analysis of all animal studies investigating MSC-EVs found high heterogeneity in how EVs are being isolated and characterized.²⁴⁷ Moreover, there were significant inconsistencies in nomenclature being used to describe EV therapy and variability in interventional characteristics including tissue source of MSCs and treatment regimen. After updating our search and limiting our review to publications related to lung disorders, we still observed similar heterogeneity. When assessing whether groups were characterizing their MSCs and EVs according to international guidelines (e.g. ISCT criteria and MISEV 2018),^{173,227} we found that 58% and 19% of studies met the recommendations, respectively. This is a significant increase from our previous reports of only 2% adherence to MISEV 2018²⁴⁷ which highlights the impact of these guidelines

for improving EV characterization. EV nomenclature remains inconsistent despite the suggestions from MISEV 2018 to describe EV therapies by physical parameters (e.g. small EVs 30-150 nm, large EVs 150-1000 nm). Based on size alone, we found that over 30% of studies are using discordant terminology. This finding may be problematic for two reasons: (1) inconsistent terms will hinder between study comparisons of different EV populations, and (2) our finding likely is an underestimate as we only considered size and did not include analysis of EV cellular origin (e.g. exosomes originate as intraluminal vesicles within late endosomes). Greater standardization in reporting of size and applying accurate terminology in the EV field can help identify specific EV populations that serve as a more effective therapeutic product.

Given the many variables involved in developing an MSC-EV therapy, we conducted exploratory subgroup analyses to determine if specific EV parameters may be associated with improved efficacy. Across studies of ALI, BPD and PAH, the different tissue sources of MSCs (e.g. bone marrow, umbilical cord, placenta) and routes of administration (e.g. intravenous, intraperitoneal, intratracheal) were equally efficacious, despite beliefs that intratracheal delivery may allow for greater lung distribution. Moreover, umbilical cord tissue is known to be more efficient in producing EVs with potentially less variability from donor demographics (e.g. age, sex, comorbidities) and serves as a non-invasive, abundant source for MSCs given its designation as biohazardous waste.^{172,443,444} Hence, the similar efficacy and ease in collection of umbilical cord tissue suggests some potential advantages when considering clinical translation. EVs isolated by TFF appeared to be less effective than those enriched by ultracentrifugation for preclinical BPD and PAH (no studies used TFF in ALI). However, these findings were from only two studies that utilized TFF and more direct comparisons of TFF and ultracentrifugation are warranted to establish the relative *in vivo* efficacy of EVs, especially given TFF's greater efficiency in EV yield and

potential for clinical scaleup.²⁴³ Lastly, we conducted subgroup analysis by EV subtype/size to investigate whether distinct EV populations may exhibit variable outcomes. Small EVs or the combination of small/large EVs were found to be more consistently beneficial as compared to large EVs in ALI and BPD (no studies used large EVs in PAH). Moreover, small EVs led to a significant improvement in lung vascular permeability as compared to large EVs. These results may indicate that there are differential effects between small and large EVs, which may not be dependent on acuity/chronicity of the disease state.

Our study had limitations which warrant discussion. Firstly, the findings from subgroup analyses should be considered exploratory. We show that specific EV characteristics are associated with greater efficacy in these investigations. However, some subgroup categories were limited by the number of available studies and/or the sample size. Hence, updated analyses when more studies are published will be required to generate definitive conclusions. Our findings identify the need for robust data from head-to-head comparisons of EV methodology and/or subtypes to identify the most effective approaches for clinical translation. Next, data generated from studies with a low risk of bias may serve as a more accurate indicator for the efficacy of MSC-EVs. Unfortunately, most studies included in our analysis demonstrated an “unclear” risk across a majority of design parameters, which is commonly observed in many fields of preclinical *in vivo* research.^{285,445-449} Future analyses will provide greater insights once researchers and journals adopt more complete reporting of preclinical study design.^{353,450,451} Lastly, although no adverse events were reported, none of the included studies conducted formal experiments to investigate MSC-EV safety.

Although MSC-EV therapy demonstrates great promise, several questions still need to be answered to advance the field. We found dose-response and biodistribution studies to be infrequently conducted in respiratory diseases, which is also the case for the MSC-EV field as a

whole.²⁴⁷ These experiments are urgently needed to better understand the optimal approach for dosing EVs before attempting clinical translation. Moreover, there are no standardized guidelines on how EV dose should be measured and reported (e.g. protein, particle or RNA amount). We now know that MSC-EVs contain many bioactive factors including mRNAs, microRNAs and proteins that modulate critical cell processes ranging from inflammation to apoptosis to angiogenesis.²⁴⁷ Although many studies show the importance of specific molecules by inhibiting their expression, few have attempted to compare whether overexpressing one bioactive factor is more important than others. Lastly, quality of reporting and potential risk of bias in study design is consistently unclear in preclinical EV research. Greater rigor and transparent detailing of experimental parameters including randomization, blinding, and sample size estimates will generate more robust evidence for EV efficacy to guide clinical translation.

4.6 Supporting Information

Supplemental File C1. PRISMA 2020 Checklist.

Supplemental File C2. Systematic Review Search Strategies.

Supplemental File C3. Supporting Figures for Study Characteristics, EV Methodology and Interventional Traits

Supplemental File C4. Meta-Analysis of Secondary Outcomes

Supplemental File C5. Subgroup Analyses of Outcomes.

4.7 Acknowledgements

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DISCLOSURE STATEMENTS

DJS is an advisor to Northern Therapeutics (Montreal, QC, Canada). The remaining authors have no competing interests to declare.

Table 4.1. Summary of study characteristics of all included articles.

First Author	Year	Country	Animal Model	Human Disease	MSC Source	EV Isolation and Size / Morphology Analysis	Terminology Consistency	Positive Surface Markers	Negative Surface Markers	Dosage Regimen (Dose, No. of Doses, Route and Immunocompatibility)
Acute Respiratory Distress Syndrome / Acute Lung Injury										
Zhu, YG	2014	United States	Mouse	Acute Respiratory Distress Syndrome	Human bone marrow	Ultracentrifugation; SEM; 150-1000 nm (large EVs)	Yes	Not described	Not described	3e6 MSC equivalent over 48h; 1 dose; Intravenous, Intratracheal; Xenogeneic
Li, L	2015	China	Mouse	Acute Respiratory Distress Syndrome	Human bone marrow	Isolation kit / PEG; Not described; Not described	Unclear	CD63	Not described	1.5ug/g; 1 dose; Intravenous; Xenogeneic
Tang, XD	2017	China	Mouse	Acute Respiratory Distress Syndrome	Human bone marrow	Ultracentrifugation; SEM, TEM; 150-1000 nm (large EVs)	Yes	Not described	Not described	30uL (equivalent to 3e6 MSCs over 48h); 1 dose; Intratracheal; Xenogeneic
Khatri, M	2018	United States	Pig	Acute Respiratory Distress Syndrome	Pig bone marrow	Ultracentrifugation; TEM; 30-150 nm (small EVs)	No	CD63, CD9, CD81, CD29 (Integrin B1/B2)	Not described	80ug/kg; 1 dose; Intratracheal; Allogeneic
Potter, DR	2018	United States	Mouse	Acute Respiratory Distress Syndrome	Human bone marrow	Ultracentrifugation; NTA; 30-1000 nm (both small/large EVs)	Yes	CD63, CD9, CD81, MSC markers	Not described	30ug; 1 dose; Not Described; Xenogeneic
Yi, X	2019	China	Mouse	Acute Respiratory Distress Syndrome	Mouse bone marrow	High speed centrifugation; NTA, TEM; 30-150 nm (small EVs)	Yes	CD63	Not described	100ug; 1 dose; Intravenous; Allogeneic
Xu, N	2019	China	Rat	Acute Respiratory Distress Syndrome	Rat bone marrow	Ultracentrifugation; NTA, TEM; 30-150 nm (small EVs)	Yes	CD63, CD9, CD81	Not described	5e6 MSC equivalent; 1 dose; Intratracheal; Allogeneic
Varkouhi, AK	2019	Canada	Rat	Acute Respiratory Distress Syndrome	Human umbilical cord	Anion-exchange chromatography; TEM; 30-150 nm (small EVs)	No	Not described	Not described	1e8 particles/kg; 1 dose; Intravenous; Xenogeneic
Silva, JD	2019	Brazil	Mouse	Acute Respiratory Distress Syndrome	Mouse bone marrow	Ultracentrifugation; NTA, DLS; 30-1000 nm (both small/large EVs)	Yes	Not described	Not described	1e5 MSC equivalent; 1 dose; Intravenous; Allogeneic
Li, JS	2019	China	Rat	Acute Respiratory Distress Syndrome	Human umbilical cord	Isolation kit / PEG; DLS, TEM; 30-150 nm (small EVs)	Yes	CD63, CD9	Not described	800 ug of RNA; 1 dose; Intravenous; Xenogeneic
Liu, J	2019	China	Rat	Acute Respiratory Distress Syndrome	Rat bone marrow	Ultracentrifugation; TEM; 30-150 nm (small EVs)	Yes	CD63, CD81, TSG101, MSC markers	Not described	5-10ug; 1 dose; Intravenous; Allogeneic
Li, QC	2019	China	Rat	Acute Respiratory Distress Syndrome	Rat bone marrow	High speed centrifugation; NTA, TEM; 30-150 nm (small EVs)	Yes	CD63, CD9, CD81	Not described	25ug; 4 doses; Intravenous; Allogeneic
Huang, R	2019	China	Mouse	Acute Respiratory Distress Syndrome	Human adipose tissue	Ultracentrifugation; NTA, TEM; 30-1000 nm (both small/large EVs)	Yes	CD63, CD81, MSC markers	Calnexin, GM130	100ug; 1 dose; Intravenous; Xenogeneic
Chen, W	2019	China	Rat	Acute Respiratory Distress Syndrome	Human umbilical cord	Ultracentrifugation; TEM; Not described	Unclear	MSC markers	Not described	Not described; 1 dose; Intratracheal; Xenogeneic
First Author	Year	Country	Animal Model	Human Disease	MSC Source	EV Isolation and Size / Morphology Analysis	Terminology Consistency	Positive Surface Markers	Negative Surface Markers	Dosage Regimen (Dose, No. of Doses, Route and Immunocompatibility)

Wang, J	2020	China	Mouse	Acute Respiratory Distress Syndrome	Human adipose tissue	Ultracentrifugation; DLS, TEM; 30-150 nm (small EVs)	Yes	CD63, CD81, CD44, CD105, CD40	Calnexin, GM130	50ug; 1 dose; Intravenous, Intratracheal; Xenogeneic
Deng, H	2020	China	Mouse	Acute Respiratory Distress Syndrome	Mouse bone marrow	Ultracentrifugation, Ultrafiltration; NTA, TEM; 30-150 nm (small EVs)	Yes	CD63, CD81	Not described	50ug or 100ug; 1 dose; Intratracheal; Allogeneic
Chen, WX	2020	China	Rat	Acute Respiratory Distress Syndrome	Human umbilical cord	Ultracentrifugation; TEM; Not described	Unclear	Not described	Not described	1e6 particles; 1 dose; Intratracheal; Xenogeneic
Asthma / Allergic Airway Inflammation										
Cruz, FF	2015	United States	Mouse	Asthma / Allergic Airway Inflammation	Human and mouse bone marrow	Ultracentrifugation; NTA, TEM; 30-1000 nm (both small/large EVs)	Yes	Not described	Not described	3e6 MSC equivalent; 1 dose; Intravenous; Xenogeneic, Allogeneic
de Castro, LL	2017	Brazil	Mouse	Asthma / Allergic Airway Inflammation	Human adipose tissue	Ultracentrifugation; DLS; 30-1000 nm (both small/large EVs)	Yes	Not described	Not described	37ug; 1 dose; Intravenous; Xenogeneic
Kim, SD	2020	South Korea	Mouse	Asthma / Allergic Airway Inflammation	Mouse adipose tissue	Ultracentrifugation, Ultrafiltration; TEM; Not described	Unclear	CD81, CD40	Not described	10ug; 4 doses; Intranasal; Allogeneic
Fang, SB	2020	China	Mouse	Asthma / Allergic Airway Inflammation	Human iPSCs	Anion-exchange chromatography; NTA, TEM; 30-150 nm (small EVs)	Yes	CD63, CD9, CD81, TSG101, Alix	Calnexin	2e10 particles; 1 dose; Intravenous; Xenogeneic
Fang, SB	2020	China	Mouse	Asthma / Allergic Airway Inflammation	Human iPSCs	Anion-exchange chromatography; NTA, TEM; 30-150 nm (small EVs)	Yes	CD63, CD9, CD81, TSG101, Alix	Calnexin	1.5e10 particles; 3 doses; Intravenous; Xenogeneic
Bronchopulmonary Dysplasia										
Ahn, SY	2018	South Korea	Rat	Bronchopulmonary Dysplasia	Human umbilical cord	Ultracentrifugation; NTA, TEM; 30-150 nm (small EVs)	No	CD63, CD9	Cytochrome C, GM130, Fibrillarin	20ug; 1 dose; Intratracheal; Xenogeneic
Willis, GR	2018	United States	Mouse	Bronchopulmonary Dysplasia	Human bone marrow, human umbilical cord	Tangential flow filtration, IDX density gradient ultracentrifugation; NTA, TEM; 30-150 nm (small EVs)	Yes	CD63, CD9, Flotillin-1	Not described	0.5e6 MSC equivalent over 36 hours; 1 dose; Intravenous; Xenogeneic
Chaubey, S	2018	United States	Mouse	Bronchopulmonary Dysplasia	Human umbilical cord	Ultracentrifugation; NTA, TEM; 30-150 nm (small EVs)	Yes	CD63, CD81, Alix	TGN48	0.7e6 MSC equivalent over 24h (2.4-2.8ug); 2 doses; intraperitoneal; Xenogeneic
Brain, RK	2018	United States	Rat	Bronchopulmonary Dysplasia	Rat bone marrow	Ultracentrifugation; NTA, TEM; 30-1000 nm (both small/large EVs)	No	CD63, CD9, CD81	Not described	15ug or 3.4e9 particles; 14 or 12 doses; intraperitoneal; Allogeneic
First Author	Year	Country	Animal Model	Human Disease	MSC Source	EV Isolation and Size / Morphology Analysis	Terminology Consistency	Positive Surface Markers	Negative Surface Markers	Dosage Regimen (Dose, No. of Doses, Route and Immunocompatibility)
Porizonato, A	2019	Italy	Rat	Bronchopulmonary Dysplasia	Human umbilical cord	Tangential flow filtration; NTA; 30-1000 nm (both small/large EVs)	Yes	CD63, CD9, CD81, Annexin V	HLAs	8e8 particles/g for P3, 4.5e8 for P7, and 3e8 for P10; 3 doses; Intratracheal; Xenogeneic

Willis, GR	2020	United States	Mouse	Bronchopulmonary Dysplasia	Human umbilical cord	Tangential flow filtration, IDX density gradient ultracentrifugation; NTA, TEM; 30-150 nm (small EVs)	Yes	CD63, CD9, CD81, TSG101, Alix, Flotillin-1	GM130	0.5e6 MSC equivalent, or 1e6 MSC equivalent; 1 or 4 doses; Intravenous; Xenogeneic
Li, Z	2020	China	Rat	Bronchopulmonary Dysplasia	Human placenta	Ultracentrifugation; TEM; 30-150 nm (small EVs)	Yes	CD63, CD9, CD81, HSP70/90	Not described	0.3ug; 1 dose; Intratracheal; Xenogeneic
Chronic Obstructive Pulmonary Disease										
Maremanda, K	2019	United States	Mouse	Chronic Obstructive Pulmonary Disease	Mouse bone marrow	Isolation kit / PEG; TRPS, TEM; 30-150 nm (small EVs)	Yes	CD63, Alix, Flotillin-1	Calnexin	15ug; 10 doses; intraperitoneal; Allogeneic
Harrell, CR	2020	Serbia	Mouse	Chronic Obstructive Pulmonary Disease	Human placenta	Ultracentrifugation, Isolation kit / PEG; Not described; Not described	Unclear	Not described	Not described	0.1mL; 15 doses; intraperitoneal; Xenogeneic
Congenital Diaphragmatic Hernia										
Zhaorigetu, S	2020	United States	Rat	Congenital Diaphragmatic Hernia	Human bone marrow	Tangential flow filtration; TRPS, TEM; 30-150 nm (small EVs)	Yes	Not described	Not described	1e10 particles/mL; 1 dose; Intravascular; Xenogeneic
Monroe, MN	2020	United States	Rat	Congenital Diaphragmatic Hernia	Human bone marrow	Tangential flow filtration; NTA, TEM; Not described	Unclear	Not described	Not described	1e10 particles/mL; 1 dose; Direct tissue injection; Xenogeneic
Ischemia-Reperfusion Injury										
Stone, ML	2017	United States	Mouse	Ischemia-Reperfusion Injury	Human umbilical cord	Ultracentrifugation; NTA; 150-1000 nm (large EVs)	No	MSC markers	Not described	1e6 particles; 1 dose; Intratracheal; Xenogeneic
Li, JW	2019	China	Mouse	Ischemia-Reperfusion Injury	Mouse bone marrow	Ultracentrifugation, Isolation kit / PEG; NTA; Not described	Unclear	CD63, CD9, CD81	Not described	30uL (2e6 MSC equivalent); 1 dose; Intratracheal; Allogeneic
Pneumonia										
Monsel, A	2015	United States	Mouse	Pneumonia	Human bone marrow	Ultracentrifugation; SEM; 150-1000 nm (large EVs)	Yes	CD44	Not described	EVs made by 9e6 MSCs over 48h; 1 dose; Intravenous, Intratracheal; Xenogeneic
First Author	Year	Country	Animal Model	Human Disease	MSC Source	EV Isolation and Size / Morphology Analysis	Terminology Consistency	Positive Surface Markers	Negative Surface Markers	Dosage Regimen (Dose, No. of Doses, Route and Immunocompatibility)
Hao, Q	2019	United States	Mouse	Pneumonia	Human bone marrow	Ultracentrifugation; NTA, SEM; 30-1000 nm (both small/large EVs)	Yes	CD44	Not described	1e10 particles; 1 dose; Intravenous; Xenogeneic
Pulmonary Arterial Hypertension										
Lee, C	2012	United States	Mouse	Pulmonary Arterial Hypertension	Mouse bone marrow	Ultracentrifugation, Size exclusion chromatography; TEM; 30-150 nm (small EVs)	Yes	CD63, CD9, CD81, TSG101, Alix, HSP70/90, Flotillin-1	Not described	0.1ug (low dose), 10ug (high dose); 1 or 2 doses; Intravenous; Allogeneic

Chen, JY	2014	United States	Rat	Pulmonary Arterial Hypertension	Rat bone marrow	Ultracentrifugation; NTA, TEM; 30-1000 nm (both small/large EVs)	No	CD29 (Integrin B1/B2), Annexin V	Not described	30ug; 8 doses; Intravenous; Allogeneic
Aliotta, JM	2016	United States	Mouse	Pulmonary Arterial Hypertension	Human and mouse bone marrow	Ultracentrifugation; NTA, TEM; 30-150nm (small EVs), 30 - 1000 nm (both EVs)	No	CD63, CD9, CD81	Not described	25ug; 3 or 4 doses; Intravenous; Xenogeneic, Allogeneic
Aliotta, JM	2017	United States	Mouse	Pulmonary Arterial Hypertension	Mouse bone marrow	Ultracentrifugation; NTA, TEM; 30-1000 nm (both small/large EVs)	Yes	CD63	Not described	25ug; 3 doses; Intravenous; Allogeneic
Liu, Z	2018	China	Rat	Pulmonary Arterial Hypertension	Rat bone marrow	Ultracentrifugation; TEM; Not described	Unclear	Not described	Not described	30ug; 8 doses; Intravenous; Allogeneic
Hogan, SE	2019	United States	Mouse	Pulmonary Arterial Hypertension	Human bone marrow	Tangential flow filtration, Size exclusion chromatography; NTA, TEM; 30-150 nm (small EVs)	Yes	CD63, CD81, TSG101, Alix, Flotillin-1, Annexin V, EpCAM, ICAM	GM130	2e7 particles in 200uL; 1 or 9 doses; Intravenous; Xenogeneic
Zhang, S	2020	China	Rat	Pulmonary Arterial Hypertension	Human umbilical cord	Ultracentrifugation; TEM; 30-150 nm (small EVs)	Yes	CD63, CD81, TSG101, Alix	Not described	25ug; 3 doses; Intravenous; Xenogeneic
Klinger, JR	2020	United States	Rat	Pulmonary Arterial Hypertension	Human bone marrow	Ultracentrifugation; NTA, TEM; 30-1000 nm (both small/large EVs)	Yes	CD63, CD9, CD81, TSG101	Albumin	100ug/kg; 3 doses; Intravenous; Xenogeneic
Pulmonary Fibrosis										
Choi, M	2014	South Korea	Mouse	Pulmonary Fibrosis	Human bone marrow	Isolation kit / PEG; Not described; Not described	Unclear	Not described	Not described	10ug; 2 doses; Intravenous; Xenogeneic
Gao, Y	2016	China	Rat	Pulmonary Fibrosis	Rat adipose tissue	Ultracentrifugation; NTA, TEM; 30-1000 nm (both small/large EVs)	Yes	CD63, TSG101, Alix	GM130	2.5-2.8e10 particles; 1 dose; Intratracheal; Allogeneic
Bandeira, E	2018	Brazil	Mouse	Pulmonary Fibrosis	Mouse adipose tissue	Ultracentrifugation; NTA, SEM, TEM; 30-1000 nm (both small/large EVs)	Yes	CD63, CD9, CD81, Lamp1	Not described	Two doses: 1) 1e5 MSC equivalent over 24h; 2) 1e6 MSC equivalent over 24h; 1 dose; Intratracheal; Allogeneic
First Author	Year	Country	Animal Model	Human Disease	MSC Source	EV Isolation and Size / Morphology Analysis	Terminology Consistency	Positive Surface Markers	Negative Surface Markers	Dosage Regimen (Dose, No. of Doses, Route and Immunocompatibility)
Mansouri, N	2019	United States	Mouse	Pulmonary Fibrosis	Human bone marrow	IDX density gradient ultracentrifugation; NTA, TEM; 30-150 nm (small EVs)	Yes	CD63, CD9, Alix, Flotillin-1	GM130	5e6 MSC equivalent; 1 dose; Intravenous; Xenogeneic
Dinh, PC	2020	United States	Rat	Pulmonary Fibrosis	Human bone marrow	Ultrafiltration; NTA, TEM; 30-150 nm (small EVs)	Yes	CD63, CD9, CD81, TSG101	Not described	10e9 particles/kg; 7 doses; Inhalation; Xenogeneic
Other Models of Lung Injury										
Lei, X	2020	China	Mouse	Radiation-Induced Lung Injury	Human placenta	Not described; NTA, TEM; 30-150 nm (small EVs)	No	CD63, CD9, TSG101, Alix	GM130	100ug/dose; 4 doses; Intravenous; Xenogeneic
Xu, B	2020	China	Rat	Smoke Inhalation Lung Injury	Rat bone marrow	Isolation kit / PEG; NTA, TEM; 30-150 nm (small EVs)	Yes	CD63, CD9, CD81	Not described	250ug; 1 dose; Intravenous; Allogeneic

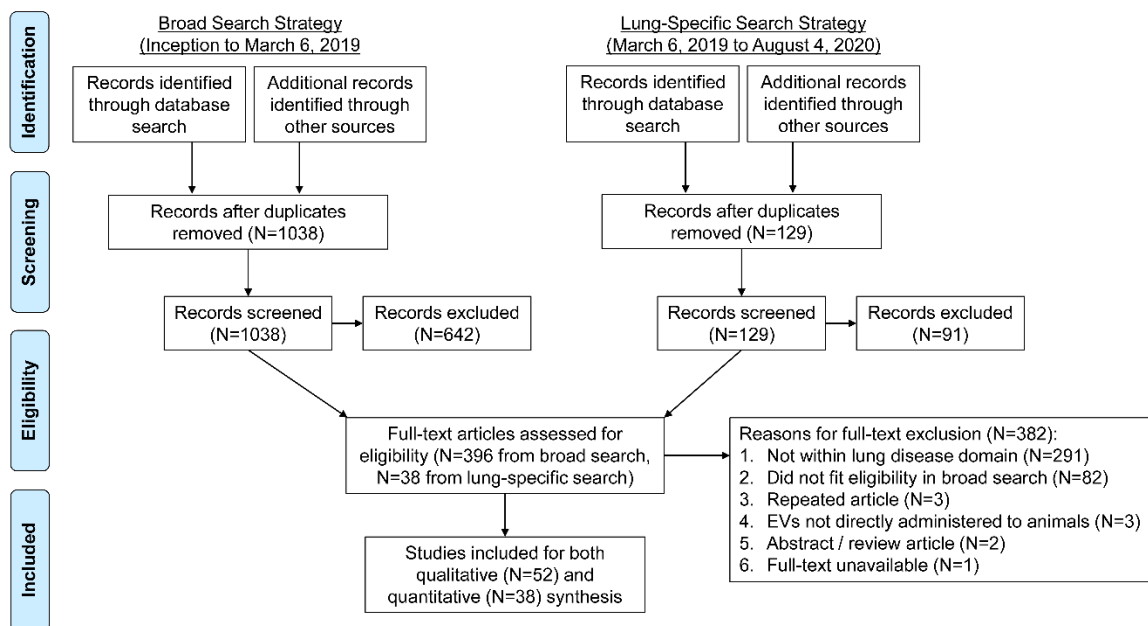


Figure 4.1. Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) flow diagram detailing study screening and selection.

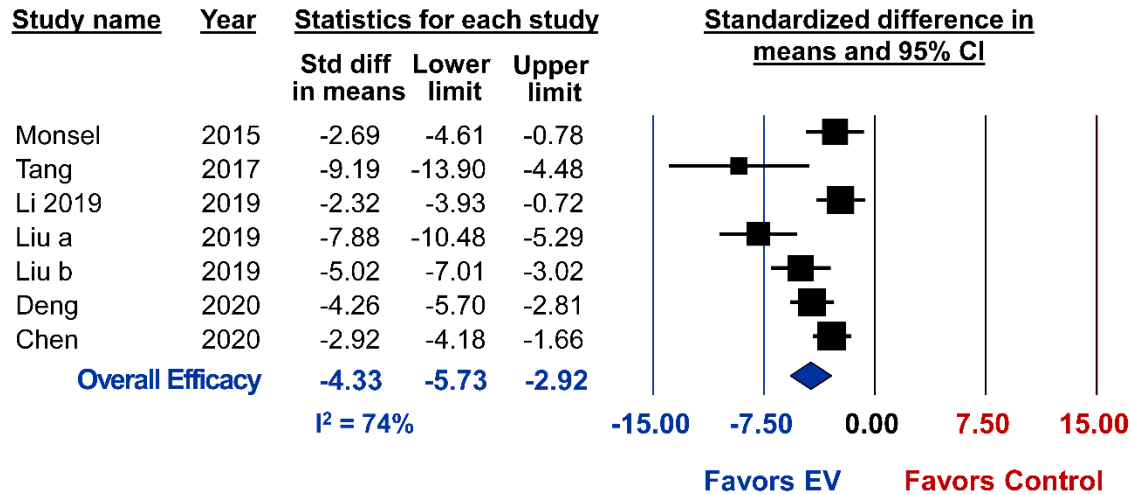


Figure 4.2. Meta-analysis of lung injure score.

Meta-analysis for all included studies of acute lung injury that reported the primary outcome of lung injure score. Data is presented as a forest plot with standardized mean difference and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect on lung injury score from all studies combined. I² value represents the statistical heterogeneity.

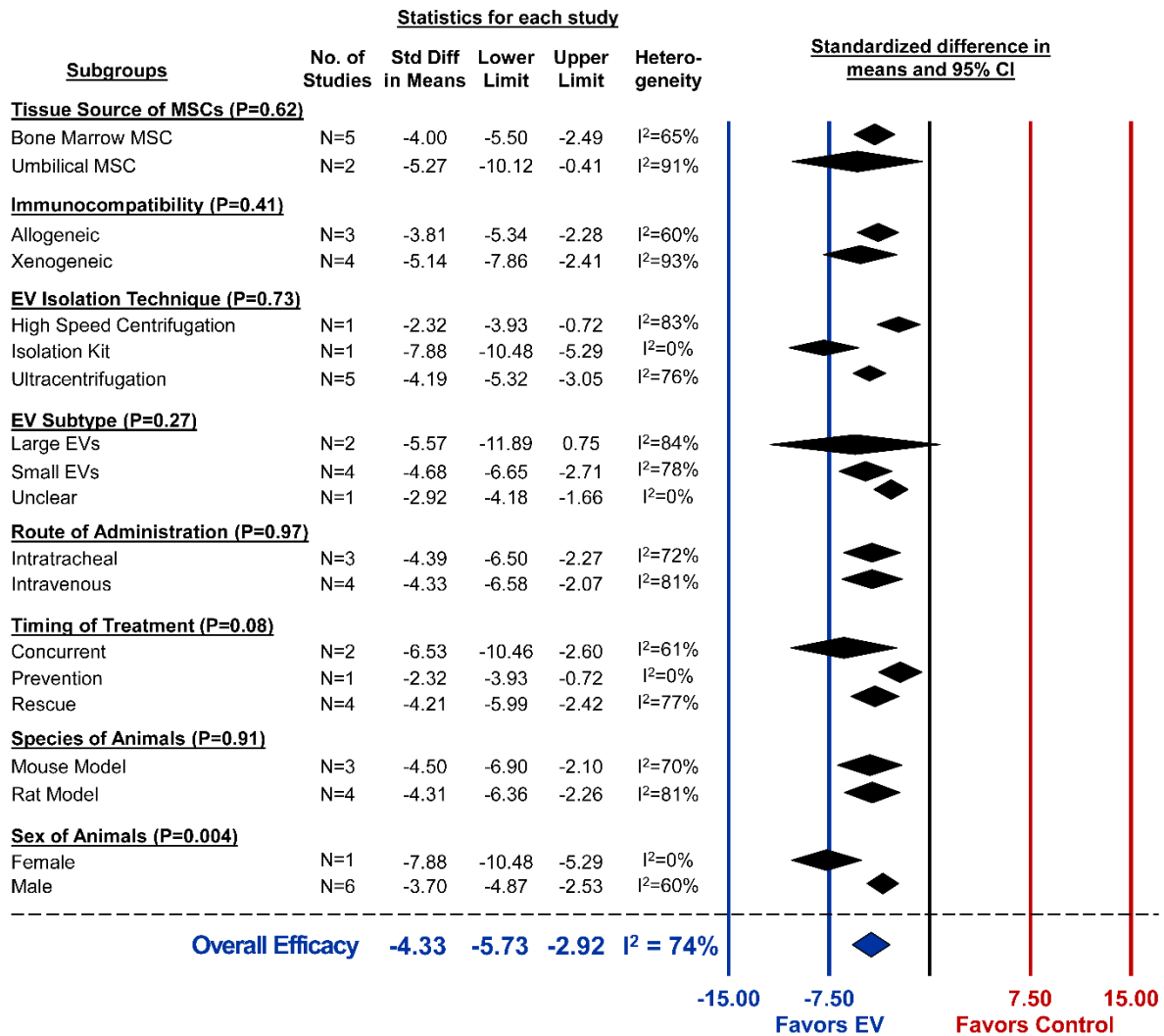


Figure 4.3. Subgroup analysis of lung injury score.

Subgroup analysis for all included studies of acute lung injury that reported the primary outcome of lung injury score. Each row represents pooled estimate data from studies within that subgroup. Data is presented as a forest plot with standardized mean difference and 95% confidence intervals. I² value represents the statistical heterogeneity within each subgroup. Effect sizes <0 favors EV treatment and >0 favors control. The ‘Overall Efficacy’ is a pooled estimate effect of MSC-EVs on lung injury score from all studies combined.

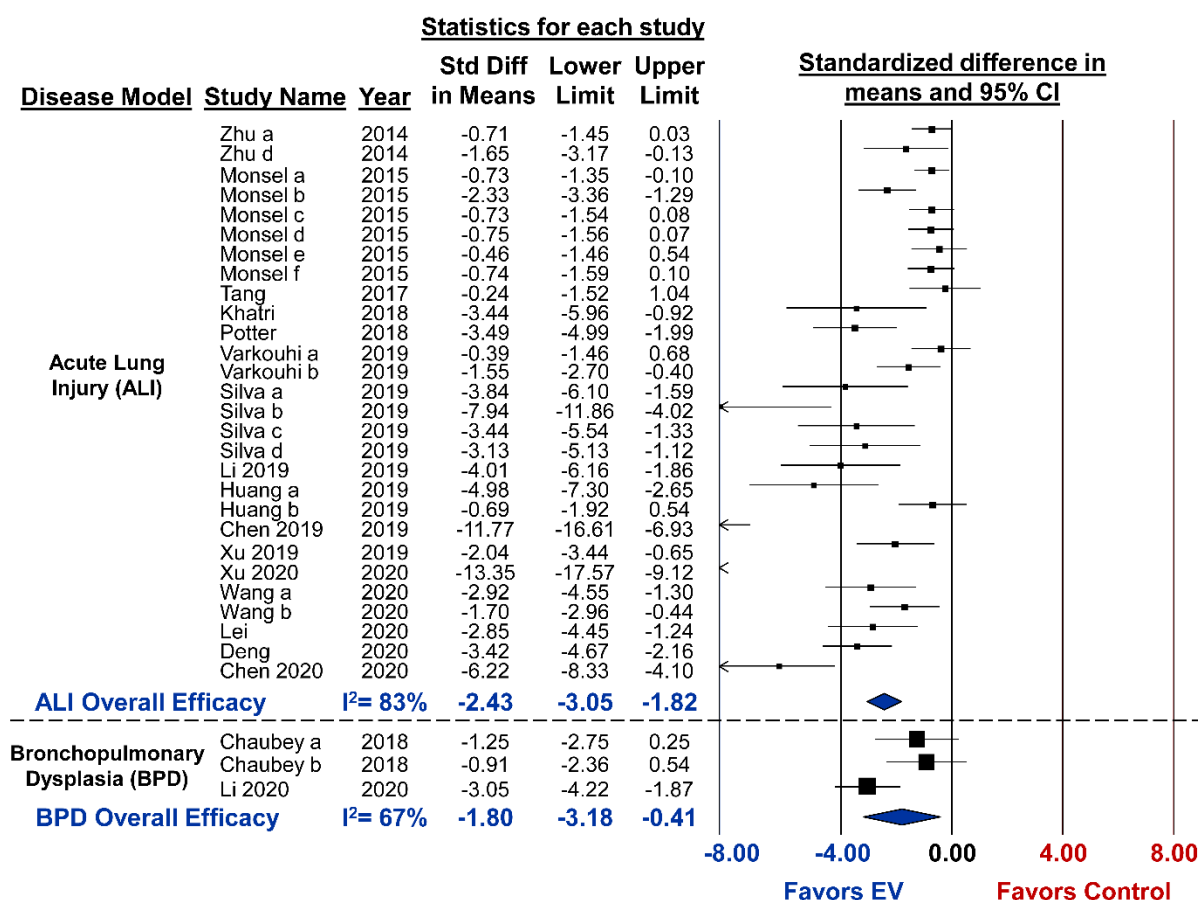


Figure 4.4. Meta-analysis of alveolar capillary permeability.

Meta-analysis for all included studies of acute lung injury and bronchopulmonary dysplasia that reported the secondary outcome of alveolar capillary permeability. Data is presented as a forest plot with standardized mean difference and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect on lung vascular permeability from all studies combined. I^2 value represents the statistical heterogeneity.

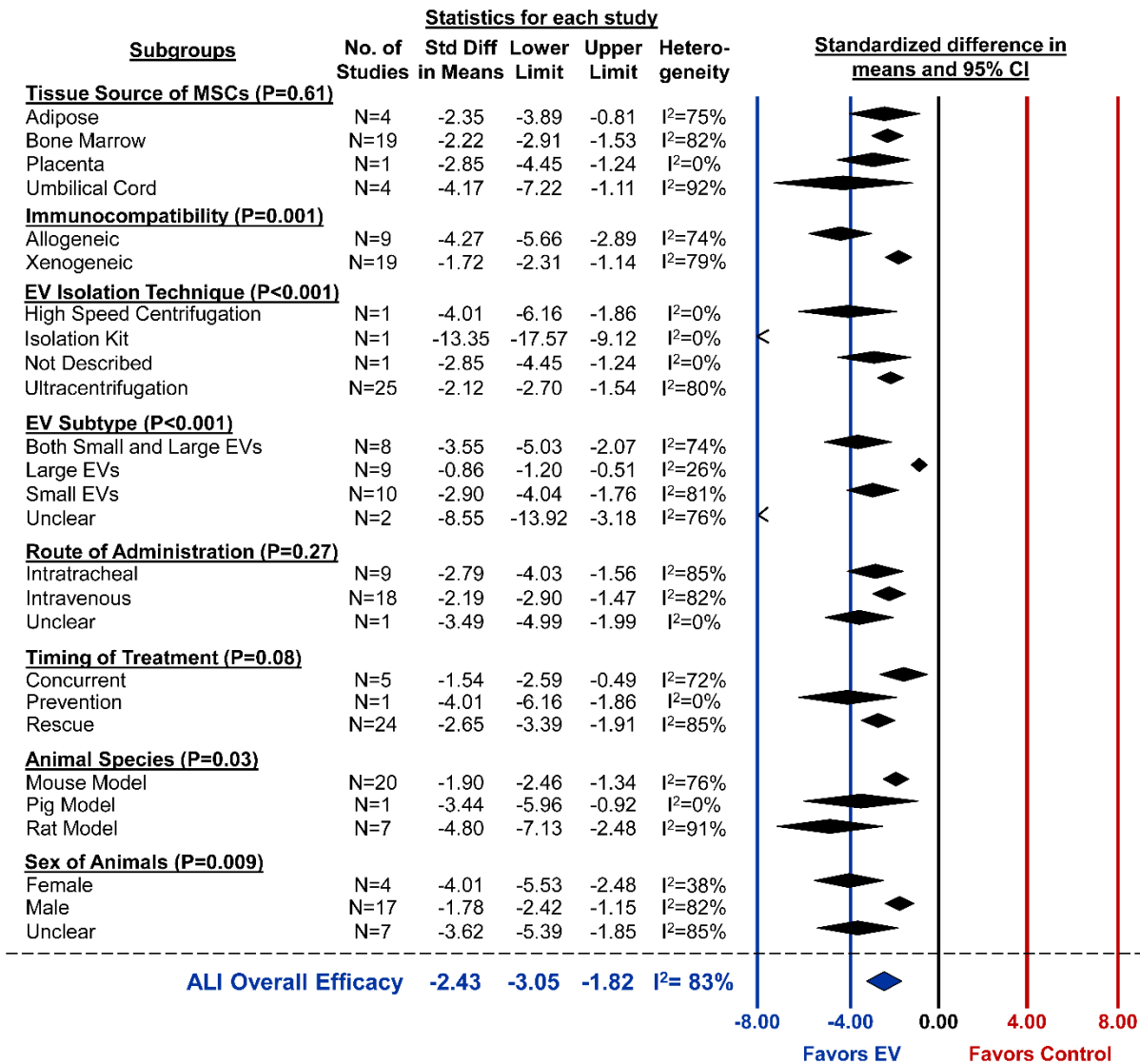


Figure 4.5. Subgroup analysis of alveolar capillary permeability.

Subgroup analysis for all included studies of acute lung injury that reported the secondary outcome of alveolar capillary permeability. Each row represents pooled estimate data from studies within that subgroup. Data is presented as a forest plot with standardized mean difference and 95% confidence intervals. I² value represents the statistical heterogeneity within each subgroup. Effect sizes <0 favors EV treatment and >0 favors control. The ‘Overall Efficacy’ is a pooled estimate effect of MSC-EVs on lung vascular permeability from all studies of acute lung injury combined.

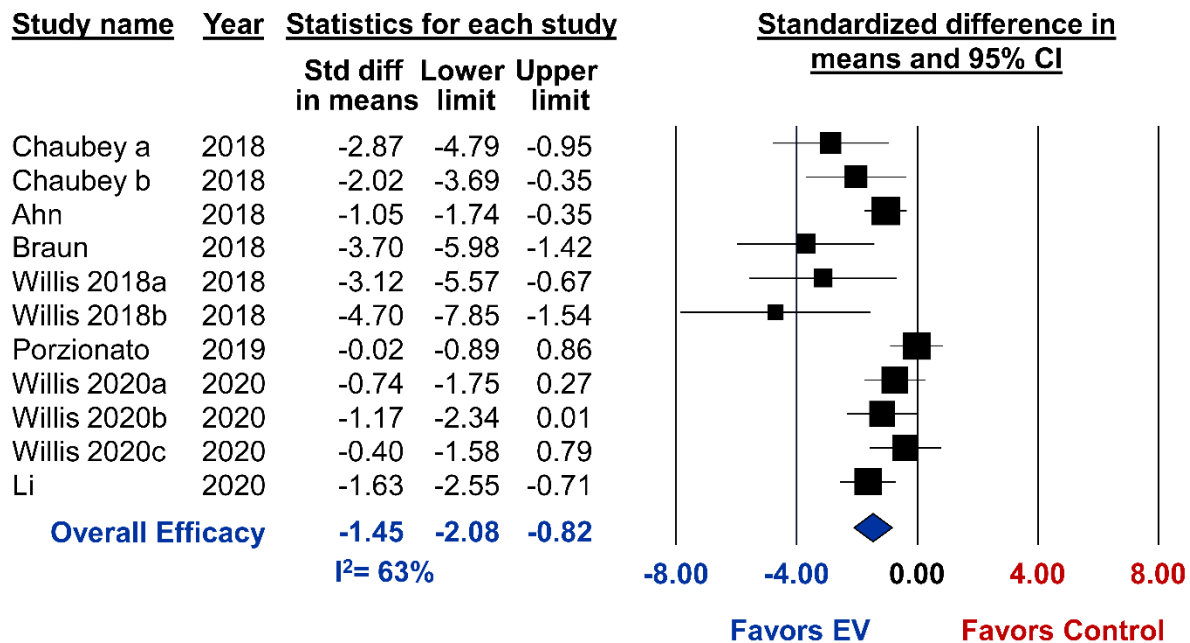


Figure 4.6. Meta-analysis of lung alveolarization.

Meta-analysis for all included studies of bronchopulmonary dysplasia that reported the primary outcome of lung alveolarization. Data is presented as a forest plot with standardized mean difference and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect on alveolarization from all studies combined. I² value represents the statistical heterogeneity.

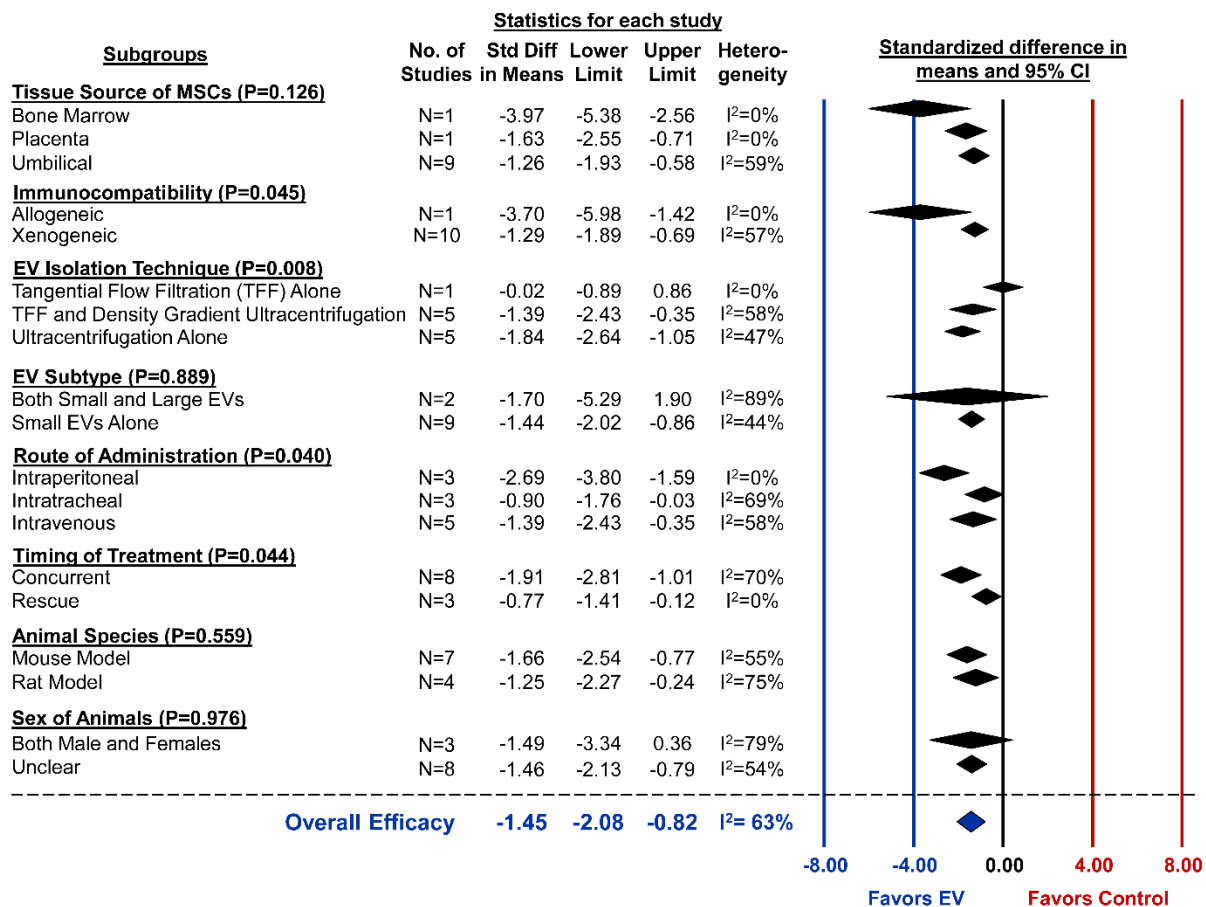


Figure 4.7. Subgroup analysis of lung alveolarization.

Subgroup analysis for all included studies of bronchopulmonary dysplasia that reported the primary outcome of lung alveolarization. Each row represents pooled estimate data from studies within that subgroup. Data is presented as a forest plot with standardized mean difference and 95% confidence intervals. I² value represents the statistical heterogeneity within each subgroup. Effect sizes <0 favors EV treatment and >0 favors control. The ‘Overall Efficacy’ is a pooled estimate effect of MSC-EVs on alveolarization from all studies combined.

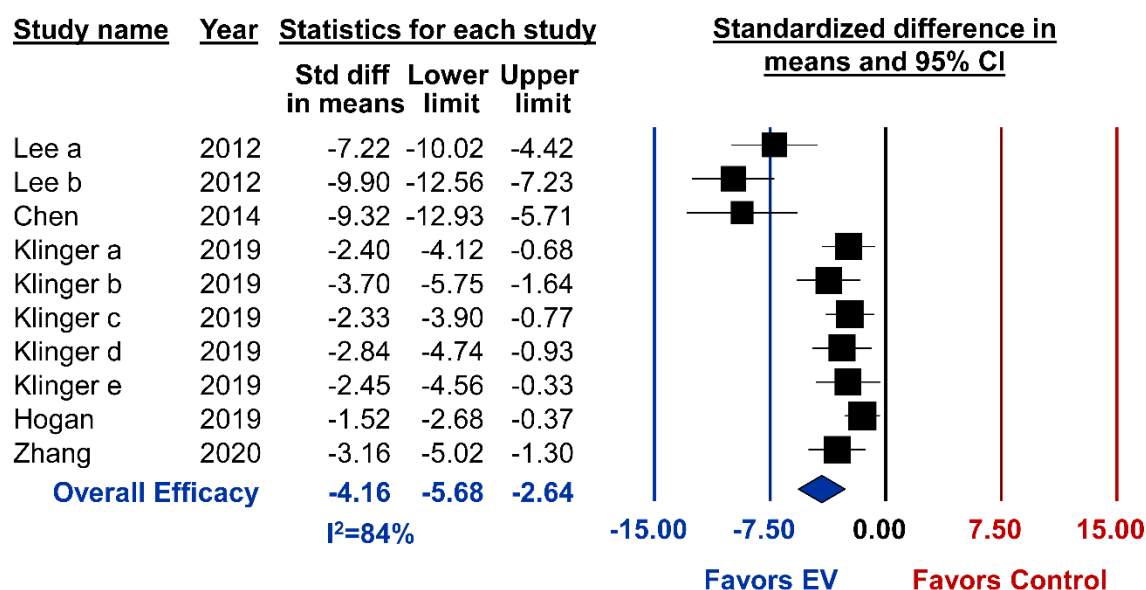


Figure 4.8. Meta-analysis of right ventricular systolic pressure.

Meta-analysis for all included studies of pulmonary arterial hypertension that reported the primary outcome of right ventricular systolic pressure. Data is presented as a forest plot with standardized mean difference and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect on right ventricular systolic pressure from all studies combined. I^2 value represents the statistical heterogeneity.

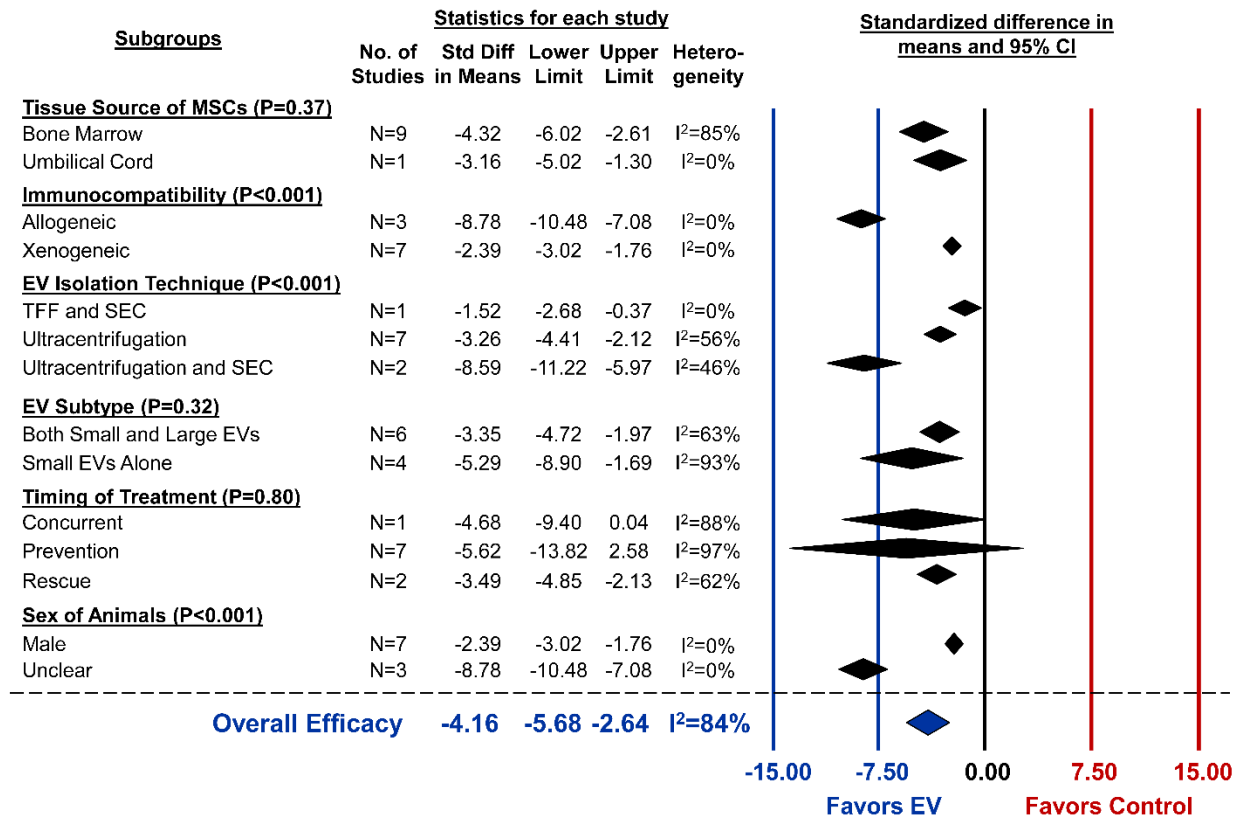


Figure 4.9. Subgroup analysis of right ventricular systolic pressure.

Subgroup analysis for all included studies of pulmonary arterial hypertension that reported the primary outcome of right ventricular systolic pressure. Each row represents pooled estimate data from studies within that subgroup. Data is presented as a forest plot with standardized mean difference and 95% confidence intervals. I² value represents the statistical heterogeneity within each subgroup. Effect sizes <0 favors EV treatment and >0 favors control. The ‘Overall Efficacy’ is a pooled estimate effect of MSC-EVs on right ventricular systolic pressure from all studies combined.

NIH Preclinical Reporting Guidelines		Level of Reporting	
Domain	Item	No. of Studies	% (n/N)
Reporting Standards	Reporting guidelines were utilized	0	100
Replicates	Results substantiated under a range of conditions	31	60 40
	Number of subjects reported for at least one <i>in vivo</i> outcome	40	77 23
	Number of subjects reported for all <i>in vivo</i> outcomes	31	60 40
	Number of measurements per subject stated for at least one <i>in vivo</i> outcome	30	58 42
	Number of measurements per subject stated for all <i>in vivo</i> outcomes	2	4 54
Statistics	Statistical tests reported	52	100
	Defined measure of central tendency	52	100
	Defined measure of dispersion	50	96 4
Randomization	Randomization of animals was reported	24	46 54
	Method of randomization was described	0	100
Blinding	Experimenters were blinded while conducting study	0	37 63
	Experimenters were blinded during outcome analysis	19	100
Sample Size Estimates	Sample size calculation was reported	7	13 87
	Adequate details provided to calculate sample size	0	100
	Total number of animals used in all studies was reported	17	33 67
Other Parameters	Primary outcome was described	0	100
	Criteria provided for exclusion of data, subjects and/or results	7	13 87

Figure 4.10. Adherence of MSC-EV studies to the NIH preclinical reporting guidelines.

Completeness of reporting for lung studies of MSC-EVs (N=52). The NIH preclinical reporting guidelines was used as the standard for reporting adherence. The absolute number of studies that reported each NIH parameter is shown. In addition, the percentage of studies that reported (green) or did not report (red) a specific study design parameter is presented.

Author, Year	Randomization	Baseline Characteristics	Allocation Concealment	Random Housing	Blinding of Personnel	Blinding of Outcome Assessment	Total No. of Animals	Selective Outcome Reporting	Other Sources of Bias:	A) Source of Funding	B) Sample Size Calculation	C) Conflict of Interest
Lee, 2012												
Chen, 2014												
Choi, 2014												
Zhu, 2014												
Li, 2015												
Cruz, 2015												
Monsel, 2015												
Aliotta, 2016												
Gao, 2016												
Willis, 2017												
Stone, 2017												
Aliotta, 2017												
de Castro, 2017												
Tang, 2017												
Ahn, 2018												
Khatri, 2018												
Potter, 2018												
Liu, 2018												
Chaubey, 2018												
Brain, 2018												
Bandeira, 2018												
Porizonato, 2019												
Li, 2019 a												
Hogan, 2019												
Yi, 2019												
Xu, 2019												
Varkouhi, 2019												
Silva, 2019												
Maremanda, 2019												
Mansouri, 2019												
Li, 2019 b												
Liu, 2019												
Li, 2019 c												
Huang, 2019												
Hao, 2019												
Chen, 2019												
Zhaorigetu, 2020												
Zhang, 2020												
Xu, 2020												
Willis, 2020												
Wang, 2020												
Monroe, 2020												
Li, 2020												
Lei, 2020												
Klinger, 2020												
Kim, 2020												
Harrell, 2020												
Fang, 2020 a												
Fang, 2020 b												
Dinh, 2020												
Deng, 2020												
Chen, 2020												

Figure 4.11. Risk of bias assessment.

Risk of bias assessment for MSC-EV studies in accordance with the SYRCLE tool. Yellow represents unclear, green represents low risk of bias and red represents high risk of bias. Light yellow represents a study that reported randomization or sample size calculations, but did not provide detailed methodology on how these parameters were achieved. Light green under “blinding of outcome” represents studies that blinded some outcomes, whereas dark green represents blinding of all outcomes.

Chapter 5 – Biodistribution of Mesenchymal Stromal Cell-Derived Extracellular Vesicles During Acute Respiratory Distress Syndrome

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Author Contributions:

AT: Devised research question, developed experimental design, conducted the animal studies, and analyzed the data. Wrote the manuscript and generated the tables/figures.

DC and CL: Research technicians. Contributed to designing/conducting experiments and revision of manuscript

DJS and MML: Principal investigators of project. Contributed to designing experiments and revision of manuscript.

5.1 Abstract

Background: Acute respiratory distress syndrome is a devastating condition characterized by severe lung inflammation. Mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs) are a promising cell-free therapy for preclinical acute lung injury (ALI, preclinical correlate of ARDSI). However, no studies have investigated their biodistribution in ALI or discerned the timing of administration for maximal lung targeting, which are crucial considerations for clinical translation. We aimed to characterize a mouse model of ALI and establish the pharmacokinetics and optimal timing for MSC-EV delivery during ALI.

Methods: MSC-EVs were isolated by ultracentrifugation or tangential flow filtration (TFF), and characterized by nanoparticle tracking analysis and western blot. A time-course study of inflammatory response over 72h was conducted using a lipopolysaccharide (LPS) induced mouse model of ALI. Severity of lung injury was assessed by histological lung injury score and analysis of cell count and inflammatory cytokines in bronchoalveolar lavage fluid. For biodistribution studies, ALI mice were intravenously injected with fluorescently labelled MSC-EVs to determine the optimal timing of administration and organ-specific biodistribution. Live *in vivo* and *ex vivo* fluorescence imaging was conducted at various timepoints post-EV injection.

Results: EVs isolated by either ultracentrifugation or TFF displayed comparable size distribution (50-350nm) and EV marker expression (CD63/81). TFF generated a 5.4-fold higher particle concentration and 3.9-fold higher total protein when compared to ultracentrifugation. From the inflammatory time-course study, cell count and IL-1 β peaked in bronchoalveolar lavage fluid at 24h after ALI induction. In subsequent administration studies, MSC-EVs delivered 24h (as

opposed to 0.5h, 5h or 10h) after disease induction produced a 2.7-4.4 fold higher aggregation of EVs to the lungs. Biodistribution studies comparing organ-specific MSC-EV uptake showed progressive lung accumulation up to 48h post-delivery (3-fold higher than the spleen/liver), with a decline at 72h. Importantly, EV fluorescence in lungs of ALI mice were significantly elevated as compared to control mice at 48h. The lung tropism of MSC-EVs was further validated as therapeutically inert EVs derived from HEK293T cells accumulated to the spleen and liver with a 5.5-fold lower degree of distribution to the lungs as compared to MSC-EVs.

Conclusion: The optimal time to administer MSC-EVs for greatest lung distribution is during heightened inflammation at 24h after ALI induction. The progressive increase in alveolar permeability and inflammatory homing signals during ALI may explain the augmented accumulation of MSC-EVs to the lungs over 48h of systemic circulation. The findings from this study provide evidence that MSC-EVs may serve as a practical rescue treatment for acute respiratory conditions due to their lung tropism, especially during states of augmented inflammation.

5.2 Introduction

Acute respiratory distress syndrome (ARDS) is a devastating critical illness with high mortality rates (30-50%).^{5,6} It carries one of the highest cost of any acute care condition due to requirements of intensive care management and prolonged hospital stay.⁴⁵² During ARDS, a localized pulmonary inflammation in response to lung infection or injury leads to vascular hyperpermeability, migration of inflammatory cells into the airways, and impaired gas exchange, all of which may induce respiratory failure. Despite decades of research, there are no curative therapies for ARDS. Preclinical animal studies have indicated mesenchymal stromal cells (MSCs) as a promising therapy to reduce inflammation, promote tissue regeneration and improve survival in ARDS.^{181,198-201} However, a recent phase IIa clinical trial for treatment of ARDS showed no differences in efficacy between MSCs and placebo control.²⁰⁷ Post-hoc analysis demonstrated a wide range in cell viability from 36 to 85%, thereby highlighting the important technical challenges faced when manufacturing an effective and viable cell therapy.

In the past decade, it has been shown that MSCs do not require tissue engraftment for efficacy.²⁰⁹⁻²¹² Rather, MSCs achieve their protective effects through paracrine mechanisms including secretion of nano-sized membrane-bound particles known as “extracellular vesicles” (EVs).^{213-217,247} These vesicles carry biologically active cargo that modulate critical cell processes including programmed cell death, proliferation and inflammation. Hence, MSC-derived extracellular vesicles (MSC-EVs) represent a possibility to harness the benefits of MSCs as an off-the-shelf, cell-free immunomodulatory therapy. In particular, respiratory diseases have become a prominent focus for the development of MSC-EVs.^{247,453} Our recent systematic review found that MSC-EV administration results in therapeutic benefit to various lung diseases ranging from acute to chronic and neonatal to adult conditions.⁴⁵⁴ Indeed, our quantitative meta-analysis demonstrated

that in animal studies of acute lung injury (ALI, preclinical correlate of ARDS), MSC-EVs improved clinically-relevant outcomes including histological lung injury, lung vascular permeability, inflammatory cell infiltration and mortality.

With rapidly rising interest in developing EV therapeutics, many studies have focused on identifying molecular mediators responsible for MSC-EV function. Of note, a variety of microRNAs, mRNAs, growth factors and transcription factors encapsulated within MSC-EVs have been shown to play critical roles in attenuating lung injury.^{247,275} The culmination of these biologically active factors enables the pro-angiogenic, pro-proliferative, and anti-inflammatory properties commonly associated with MSC-EVs.²⁴⁷ Although these mechanistic insights add to our growing understanding by which EVs exert their benefits, the tissue distribution pattern of MSC-EVs has yet to be well characterized. We previously reported that less than a quarter of MSC-EV studies conducted biodistribution analyses²⁴⁷ and our lung-focused systematic review of MSC-EVs found only two studies that qualitatively observed EV distribution in histological lung sections by fluorescence microscopy.^{269,382} Neither study quantitatively assessed the biodistribution of MSC-EVs during ALI, nor did they determine optimal the timing of administration for maximal lung targeting. In other disease states, the distribution pattern of MSC-EVs has been linked to regions of injury where pro-inflammatory environments may facilitate vesicle homing.⁴⁵⁵⁻⁴⁵⁷ Here, we used fluorescently labelled MSC-EVs and *in vivo* optical imaging techniques to establish the pharmacokinetics and optimal timing of MSC-EV delivery in a preclinical mouse model of ALI. We hypothesized that, during a state of heightened lung vascular permeability and inflammation, MSC-EVs would exhibit greater targeting to the lung tissue.

5.3 Materials and Methods

5.3.1 Study Approval

Animal study protocols were approved by the Animal Care Committee of the University of Ottawa.

5.3.2 Cell Culture

Human umbilical cord MSCs (UC-MSCs) were derived from umbilical cords as previously described.⁴⁵⁸ Both human bone marrow MSCs (BM-MSCs, Lonza, Basel, Switzerland) and umbilical cord MSCs (UC-MSCs, University of Dresden) ranging from passage 3-6 were cultured in 175cm² flasks for production of EVs. BMSCs and UC-MSCs were cultured in Minimum Essential Medium α (MEM- α , Thermo Fisher Scientific, MA, US) supplemented with 20% fetal bovine serum (FBS, Thermo Fisher Scientific, MA, US) and penicillin/streptomycin. MSCs were confirmed for their adherent property to plastic during culture conditions and tri-lineage differentiation potential. As well, flow cytometry (Attune Acoustic Focusing cytometer, Invitrogen, CA, US) was used to detect the expression (CD105, CD73, CD90) and absence (CD19, CD34, CD14, and HLA-DR) of surface antigens, which was analyzed using FlowJo 10.0 software (FlowJo, LLC).⁴⁵⁹

HEK293T cells (ATCC, VA, USA) were cultured with Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, MA, US) supplemented with 10% FBS and penicillin/streptomycin in 175cm² flasks. All cell cultures were maintained in a 37°C, 5% CO₂ and 5% O₂ tissue culture chamber.

5.3.3 EV Isolation and Characterization

MSCs and HEK293Ts at 80-90% confluency were cultured for 24h with serum-free media. The media was collected centrifuged at 2,500g for 10mins at 4°C to remove live cells and apoptotic

bodies. The supernatant was collected and frozen at -80°C until EV isolation. After media collection, cells were counted using an automated cell counter (Countess™, Thermo Fisher Scientific, MA, US, or CellDrop™, DeNovix, DE, USA).

Two techniques were employed for EV isolation: (1) *Ultracentrifugation*: Conditioned media was centrifuged at 20,000g for 20 mins. The supernatant was collected and spun at 100,000g for 90mins at 4°C (Optima L-100 XP Ultracentrifuge, Rotor 45Ti, Beckman Coulter, CA, USA) and then resuspended into phosphate buffered saline (PBS, Thermo Fisher Scientific, MA, USA). A second 100,000g spin (OptimaMAX Ultracentrifuge, Rotor TLA55, Beckman Coulter, CA, USA) was completed before resuspending into PBS for storage at -80°C. (2) *Tangential Flow Filtration (TFF)*: Supernatant was concentrated using a modified polyethersulfone hollow fiber with a 500 kDa filter cartridge (MidGee Hoop Ultrafiltration Cartridge, UFP-500-C-H42LA, GE Healthcare, UK), followed by centrifugation at 100,000g for 30 mins at 4°C before resuspension in PBS and storage at -80°C.

Protein concentration of EVs was determined by DC protein assay (Bio-Rad, ON, Canada) and immunoblot analysis of CD63 and CD81 were used to confirm EV enrichment. Size distribution and particle concentration was determined by ZetaView (Particle Metrix, Meerbusch, Germany).

5.3.4 Immunoblot

An SDS-polyacrylamide gel electrophoresis of MSC-EVs (5 µg) was performed with 4–20% Mini-PROTEAN® TGX™ Protein Gels (Bio-Rad, ON, Canada). Separated proteins were transferred to nitrocellulose membranes (NOVEX iBLOT Transfer Stacks, Thermo Fisher Scientific, ON, Canada) and membrane blots were blocked with a 5% milk solution in TBS-T (tris-buffered saline with 0.1% Tween-20). After blocking, blots were incubated overnight at 4°C with

primary antibodies for EV proteins including CD63 (1:200, Abcam, Canada) and CD81 (1:200, Santa Cruz, USA). Washed membranes were incubated with an IRDye® 800CW anti-rabbit secondary antibody (LI-COR Biotechnology, NE, USA) before imaging with the Odyssey® imaging system (LI-COR Biotechnology, NE, USA).

5.3.5 Mouse Model of Acute Lung Injury

C57BL/6 male mice (20–25g) aged 8–12 weeks were used for all animal studies. Mice were anesthetized with ketamine/xylazine, orally intubated with a sterile catheter and intratracheally administered 50µg of lipopolysaccharide (LPS, *E. coli* 055:B5; MilliporeSigma, VT, USA) resuspended in 40uL of PBS. For studies assessing MSC-EV biodistribution, mice received jugular vein injections of either PBS (background control) or DiR labelled MSC-EVs. The incision site was sutured after EV infusion and animals were assessed twice daily for wellness.

At study endpoint in the inflammatory time-course experiment, the left lung was collected for histological analysis. The right lung was lavaged three times with 500µL of saline to collect bronchoalveolar lavage fluid (BALF) for outcome analysis. Blood was collected through the inferior vena cava and centrifuged at 2000g for plasma collection, and subsequently stored at -80°C.

5.3.6 Lung Histology

Two separate sections of the left lung were used for histopathological analysis. Lung tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sliced into 5 µm thick sections with a microtome (Leica Microsystems, Ontario, Canada). Lung sections were adhered to a microscope slide and stained with hematoxylin and eosin. Images were acquired by Panoramic DESK (3DHISTECH, Hungary) scanscope using a Panoramic Scanner and analysed using CaseViewer (3DHISTECH, Hungary). Five random high-power fields (40x magnification) for

each mouse were analyzed for histological lung injury and scored as per the American Thoracic Society guidelines for features and measurements of experimental ALI.⁵⁷

5.3.7 Optical Imaging

The IVIS Spectrum *in vivo* imaging system (PerkinElmer Inc., Waltham, MA, USA) was utilized to investigate the biodistribution of MSC-EVs and EVs derived from HEK293T cells (HEK-EVs). EVs were labelled with a lipophilic near-infrared dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR; Invitrogen, CA, USA) according to manufacturer's recommendations. Briefly, EVs are incubated with 1 μ M of DiR for 15 minutes followed by 100,000 g ultracentrifugation at 4°C before resuspension in PBS. Mice received 15 μ g of DiR labelled EVs via jugular vein injection. Fluorescence imaging was conducted using the IVIS Spectrum system with excitation and emission filters at 745nm and 800nm, respectively. To measure the biodistribution of DiR labelled EVs, regions of interest were gated around *ex vivo* organs. Fluorescence was measured in units of radiant efficiency, which is the ratio of emission light (photons/sec/cm²/sr) to excitation light (μ W/cm²). In all experiments and at each timepoint, mice treated with PBS were used to generate background fluorescence that was subtracted from EV-treated organ fluorescence values.

5.3.8 Measurements of BALF Cell Count, Protein Concentration and Cytokine Analysis

BALF cell concentration was determined using a CountessTM automated cell counter (Thermo Fisher Scientific, MA, USA). BALF was then centrifuged at 800g to pellet cells, while the supernatant was frozen at -80°C until further analysis.

BALF protein concentration was measured using a DC protein assay (Bio-Rad, ON, Canada). IL1- β and IL-6 concentrations in BALF and plasma were measured using murine cytokine-specific ELISA kits (Thermo Fisher Scientific, MA, USA).

5.3.9 Statistical Analysis

Data are represented as mean $\pm$ SEM unless otherwise stated. Statistical analysis was completed using Student's t-test or One-Way ANOVA (> 2 groups) followed by Tukey's post-hoc test for multiple comparisons. All statistical analyses were conducted using GraphPad Prism (GraphPad Software, version 9.1.0, San Diego, USA). P-value < 0.05 was considered significant.

5.4 Results

5.4.1 Characterization of MSC-EVs by Tissue Source and Isolation Method

Bone marrow, the most common source for MSC-EV enrichment, requires an invasive procedure that may not be the most efficient for MSC isolation.²⁴⁷ Conversely, umbilical cord tissue has emerged as an attractive, non-invasive source given its abundance and efficiency in generating MSCs.^{172,442} Hence, we first determined which MSC source may be most effective for EV production. As compared to bone marrow MSCs, UC-MSCs exhibited more typical MSC morphology including flattened spindle shape enabling increased cell density (**Figure 5.1A**). EVs from both tissue sources displayed CD63/CD81 expression by Western Blot (**Figure 5.1B**). Although umbilical cord-derived EVs displayed the largest mean size (112.2 ± 52.3 nm), both group of EVs were within the 30-150nm size range (**Figure 5.1C**). UC-MSCs produced a concentration of EVs 3-fold higher than BM-MSCs. Hence, UC-MSCs were selected as the cell type of choice for *in vivo* biodistribution experiments.

Conditioned media from UC-MSCs were isolated by both ultracentrifugation and TFF to compare their isolation efficiency. Both methods isolated EVs with comparable size distribution (**Figure 5.1D**). TFF resulted in 5.4-fold more EV particles and 3.9-fold higher total protein as

compared to ultracentrifugation. Hence, TFF was used as the primary isolation technique for subsequent experiments.

5.4.2 UC-MSCs Have Markedly Elevated EV Production as Compared to HEK293T Cells

HEK293T cells have been widely used in EV research to their rapid proliferation, high yield and ease of genetic manipulation.^{280,460,461} Efficiency of EV production between UC-MSCs and HEK-EVs were compared (N=4 per group). On average, 4.9 ± 0.4 million UC-MSCs in a 175cm^2 flask reach confluency for media collection and EV isolation. In the same flask, 12.1 ± 0.2 million HEK293T cells reach confluency for EV isolation. When utilizing TFF for EV isolation, UC-MSCs produce 0.46 ± 0.02 μg of EV protein per mL of media. In comparison, HEK293Ts produce 0.24 ± 0.02 μg of EV protein per mL of media. Normalizing to 1 μg EVs produced, UC-MSCs (0.68 ± 0.07 million cells/ μg) were over 4-fold more efficient at producing EVs than HEK293Ts (2.95 ± 0.15 million cells/ μg).

5.4.3 Molecular Markers of Inflammation Peak at 10-24h After ALI Induction

We conducted a time-course study to determine the inflammatory response in our mouse model of ALI. Following intratracheal LPS administration, mice were sacrificed at 1h, 3.5h, 10h, 24h and 72h. In plasma, variable concentrations in IL-1 β and IL-6 were observed with a potential peak in IL-6 at 3.5h post-LPS (**Figures 5.2A and B**). Lung vascular permeability as measured by BALF protein concentration was maximally detected at 24 to 72 hours after disease induction (**Figure 5.2C**). BALF cell count and IL-1 β levels were highest at 24h post-LPS, whereas IL-6 levels peaked at 10h (**Figures 5.2D-E**). These results indicate a localized pulmonary injury from intratracheal LPS administration with a peak in inflammatory response at 10-24 hours after disease induction. A delayed response in histopathological lung injury was observed as lung injury score was highest at 72 hours after LPS administration (**Figure 5.3**).

5.4.4 Optimal Timing of MSC-EV Administration During ALI

MSC-EVs were intravenously injected into mice at 0.5h, 5h, 10h and 24h after ALI induction (**Figure 5.4A**). Given the animal skin/tissue barrier and autofluorescence of rodent chow,^{462,463} live fluorescence images of mice were assessed qualitatively (**Figure 5.4B**). The fluorescence intensity of *ex vivo* lungs and livers enabled quantitative comparison of MSC-EV distribution between groups. EVs injected 24h post-LPS, at the height of lung inflammation and alveolar permeability, resulted in enhanced accumulation compared to other administration times assessed (**Figure 5.4B**). In contrast, time of EV administration did not affect the hepatic distribution of MSC-EVs as no inflammatory changes or hyperpermeability within the liver was expected (**Figure 5.4C**). Hence, the 24h administration timepoint was used for subsequent experiments.

5.4.5 Biodistribution Analysis Indicates Lung Tropism by MSC-EVs

We next aimed to better understand the kinetics and organ-specific uptake of EVs over time. ALI was induced in mice through intratracheal LPS administration at 0h, followed by delivery of DiR labelled MSC-EVs (15µg) at 24h. Mice were allocated into groups and underwent live and *ex vivo* organ imaging at 0.5h, 3h, 10h, 24h, 48h or 72h after EV administration (**Figure 5.5A**). From live mice imaging, greater fluorescence in the lung and liver region was evident over 72h as EVs were given more time to redistribute (**Figure 5.5B**). From quantitative *ex vivo* organ analysis, we found progressive increase in lung accumulation over 72h after MSC-EV injection with maximal accumulation at 48h. Importantly, MSC-EV fluorescence intensity in the lungs of mice with ALI at 48h was 3-fold higher than the spleen and liver, and 80-fold higher than kidneys (**Figure 5.5C**).

Next, we administered DiR labelled MSC-EVs in naïve mice to determine whether the enhanced lung accumulation of MSC-EVs is due to a state of pulmonary inflammation and hyperpermeability. In contrast to ALI, naïve mice demonstrated markedly reduced distribution to the lungs with greater accumulation to the liver and spleen at 48h as compared to mice with lung injury (**Figure 5.6**). These findings confirm that MSC-EVs exhibit augmented lung tropism during ALI.

5.4.6 Biodistribution of HEK-EVs Indicate High Liver and Spleen Targeting

To investigate whether the lung tropism of EVs is specific to MSC-EVs, DiR labelled HEK-EVs (15µg) were intravenously injected into mice at 24h after ALI induction (**Figure 5.7A**). Similar to MSC-EVs, HEK-EVs displayed markedly elevated lung targeting during ALI as compared to naïve mice (**Figure 5.7B**). No differences in HEK-EV accumulation to the liver or spleen were observed between ALI or naïve mice (**Figures 5.7C and D**). Notably, HEK-EVs exhibited a 5.5-fold lower distribution to the lungs as compared to MSC-EVs at 48h, and a 3.1-fold and 7.7-fold greater distribution to the liver and spleen, respectively (**Figure 5.8**).

5.5 Discussion

The field of EV therapeutics is growing at an exponential rate due to their favorable properties as an off-the-shelf, cell-free intervention. Hundreds of articles have provided evidence for the restorative abilities of MSC-EVs in diverse disease domains. However, our preclinical systematic review investigating the efficacy of MSC-EVs found a paucity of biodistribution experiments to confirm whether EVs do in fact reach their target tissue of interest.²⁴⁷ Within the respiratory field domain, no existing publications have examined the distribution kinetics of MSC-EVs during pulmonary disease.⁴⁵⁴ In this study, we first characterized an LPS-induced mouse

model of ALI. Peak inflammatory response and vascular permeability was detected 10-24 hours after intratracheal LPS. Notably, delivering MSC-EVs at 24 hours post disease induction resulted in enhanced lung accumulation of EVs. To our knowledge, no other studies have investigated the most effective timing of EV administration during ALI. Notably, these findings align well with clinical expectations as MSC-EV therapies may be administered as a rescue intervention for patients with ARDS, while still exhibiting lung localization.

It is known that *in vivo* biodistribution of EVs reflects the cell source. Moreover, the main sites of MSC-EV accumulation have been reported to be in the liver and spleen, with minimal distribution to the lungs.⁴⁵⁷ Interestingly, we found that umbilical cord MSC-EVs exhibited high lung tropism that was far greater than any other organ, which was further pronounced during ALI. These findings can be attributed to the increase in lung vascular permeability as well as the immunomodulatory properties and inflammatory homing often associated with MSC-EVs.⁴⁵⁷ For example, EVs exhibit negligible accumulation to the heart and kidneys in healthy animals.⁴⁶⁴ However, in a mouse model of myocardial infarction, deposition of MSC-EVs into the infarcted heart was markedly elevated as compared to healthy animals due to increased coronary vascular permeability.⁴⁶⁵ Similarly, preferential targeting of MSC-EVs to the kidneys was observed in response to acute renal injury.⁴⁵⁵ Proteomic profiling of macrophage-derived EVs revealed integrin expression as a large proportion of exosomal membrane proteins.⁴⁶⁶ These cell adhesion molecules were found to be integral in the selective binding of EVs to the site of injury during kidney ischemia/reperfusion, which was otherwise negligible in healthy animals. Authors also found that EV accumulation was gradually increased with prolonged ischemic injury.⁴⁶⁶ Similarly in our study, we observed a peak in EV distribution at 48h after delivery (72h after disease induction), which corresponds to a peak in histological lung injury.

Interestingly, when the biodistribution of HEK-EVs were evaluated, we found that lung tropism was specific to MSC-EVs as HEK-EVs accumulated in the liver and spleen with minimal distribution to the lungs. A recent systematic review that synthesized EV biodistribution data illustrates that this pattern of HEK-EV organ disposition is consistent with what is commonly observed in EVs from various cell sources.⁴⁶⁴ The liver and spleen are known to be common sites for clearance of EVs. Moreover, given HEK293T cells are therapeutically inert unlike MSC-EVs, the preferential targeting of HEK-EVs to injured lungs during ALI was also significantly attenuated. These findings add to the growing notion that MSC-EVs may serve as a practical intervention to disseminate to the lungs for exertion of their immunomodulatory effects during ALI.

Our study observed a more efficient production of EVs when utilizing TFF combined with umbilical cord MSCs as opposed to ultracentrifugation and bone marrow tissue. Ultracentrifugation is the most frequently applied technique for EV enrichment due to its widespread availability and isolation specificity.²⁴⁷ However, potential aggregation of proteins, limitations on the amount of material that can be processed, and its inefficient yield of EVs are considerable limitations to clinical scaleup.^{236,238} More recently, TFF has become an appealing approach with its minimal restrictions on media volume and absence of centrifugal force that prevents vesicle damage.²⁴⁷ Previous articles have similarly demonstrated markedly improved EV yield, purity and batch-to-batch consistency, in addition to potentially greater efficacy from TFF isolation.^{243,467}

There are limitations in our study that warrant discussion. Although we did not conduct an interventional *in vivo* study, the therapeutic efficacy of MSC-EVs in preclinical ALI have been well documented and our recent meta-analysis of these studies provide further evidence for their

protective properties.⁴⁵⁴ Due to the scarcity of pharmacokinetic insight, we decided to focus our efforts on addressing the important knowledge gaps of EV biodistribution and timing of administration during respiratory injury. Of note, we used DiR, a lipophilic membrane dye, for our imaging studies. While there may be alternative methods that offer increased specificity including membrane radiolabeling or engineering with bioluminescent tags, DiR offers high sensitivity during *ex vivo* organ imaging, efficient EV labelling and simplicity with no requirements of molecular modifications.⁴⁶⁸ Moreover, fusion of bioluminescent tags to EV proteins (e.g. CD63) has been shown to alter the physiological biodistribution of EVs which may produce findings that is not representative of unaltered MSC-EV pharmacokinetics.⁴⁶⁸

5.6 Conclusion

In summary, we found that intravenous delivery of MSC-EVs at 24 hours after the onset of respiratory injury allows for maximal lung accumulation. Notably, MSC-EVs most commonly distribute to the lungs, followed by the liver and spleen with negligible accumulation to the kidneys. MSC-EVs derived from human umbilical cord tissue exhibit enhanced lung tropism, which is even more pronounced during ALI. This finding was further validated as EVs derived from therapeutically inert HEK-239T cells demonstrated a markedly attenuated lung distribution pattern with greater accumulation to the liver and spleen. Overall, the preferential distribution of MSC-EVs to the lungs during ALI when delivered as a rescue therapy represents a promising finding to optimize treatment protocol and attenuate lung injury during ARDS.

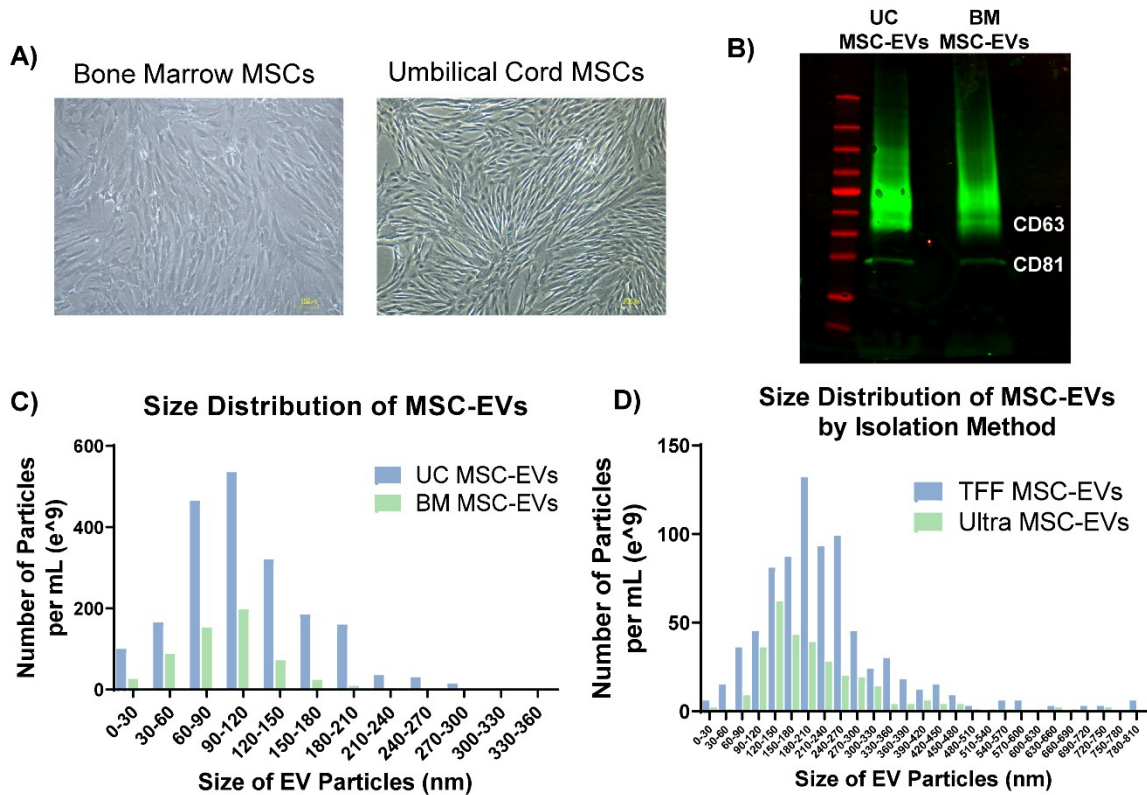


Figure 5.1. MSC-EV isolation and characterization.

(A) Comparison of cell morphology between bone marrow (BM) and umbilical cord (UC) derived MSCs. (B) Western Blot analysis of EV protein markers including CD63 and CD81 from both sources of MSC-EVs. (C) Comparison of particle size distribution between UC MSC-EVs (blue) and BM MSC-EVs (green). (D) Comparison of particle size distribution between UC MSC-EVs isolated by tangential flow filtration (TFF) and ultracentrifugation (green).

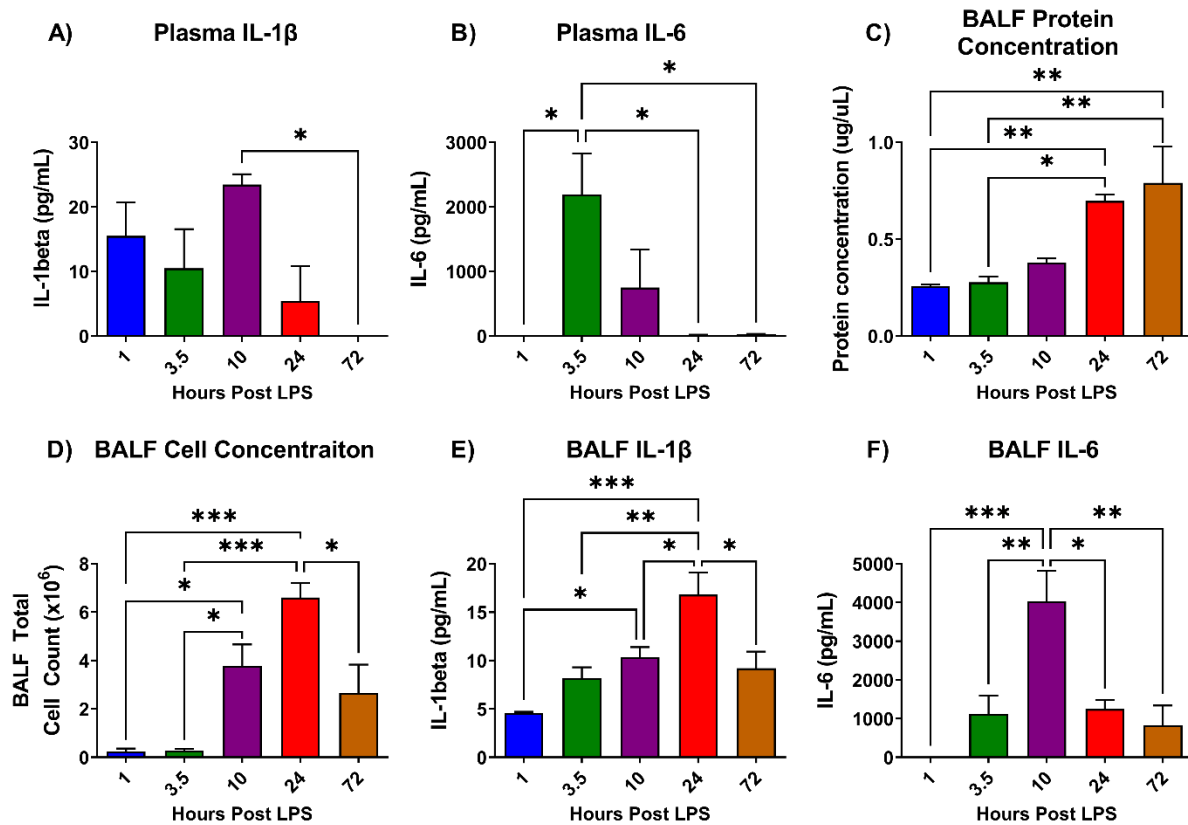


Figure 5.2. Inflammatory time course study of LPS induced mouse model of ALI.

Mice were sacrificed at various timepoints after intratracheal LPS. Plasma concentrations of (A) IL-1 β and (B) IL-6 were variable after disease induction. (C) Lung vascular permeability as assessed by bronchoalveolar lavage fluid (BALF) protein concentration peaked at 24 to 72 hours. Both BALF (D) cell concentration and (E) IL-1 β levels were highest at 24 hours after ALI induction, whereas (F) IL-6 levels peaked at 10 hours. Data are presented as mean $\pm$ SEM, N=3-4 per timepoint, * P<0.05, ** P<0.01, *** P<0.001.

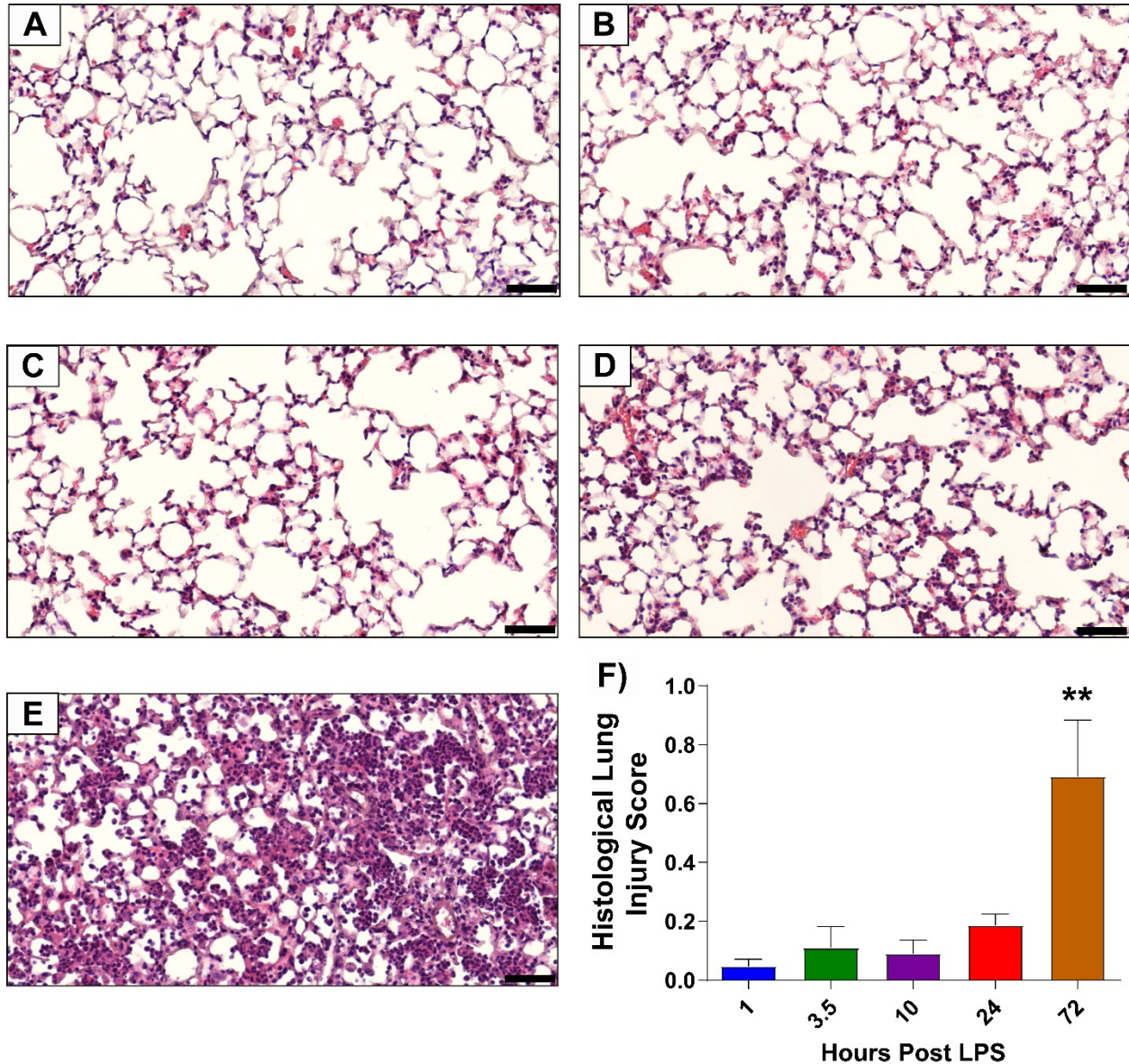


Figure 5.3. Histological analysis of mice lung sections at various timepoints after LPS-induced ALI.

Representative histological images are shown for 1h, 3.5h, 10h, 24h and 72h (panels A-E, respectively). (F) Histological lung injury score was highest at 72h and evaluated as per the American Thoracic Guidelines, which includes assessment for: (1) Intra-alveolar cell infiltration, (2) cell infiltration into interstitial space, (3) hyaline membranes, (4) proteinaceous debris in airspace (e.g. fibrin strands), and (5) alveolar septal thickening.⁵⁷ N=3-5 per dose. Data are presented as mean±SEM with a maximum score of 1.0, ** P<0.01. Scale bar represents 50 µm.

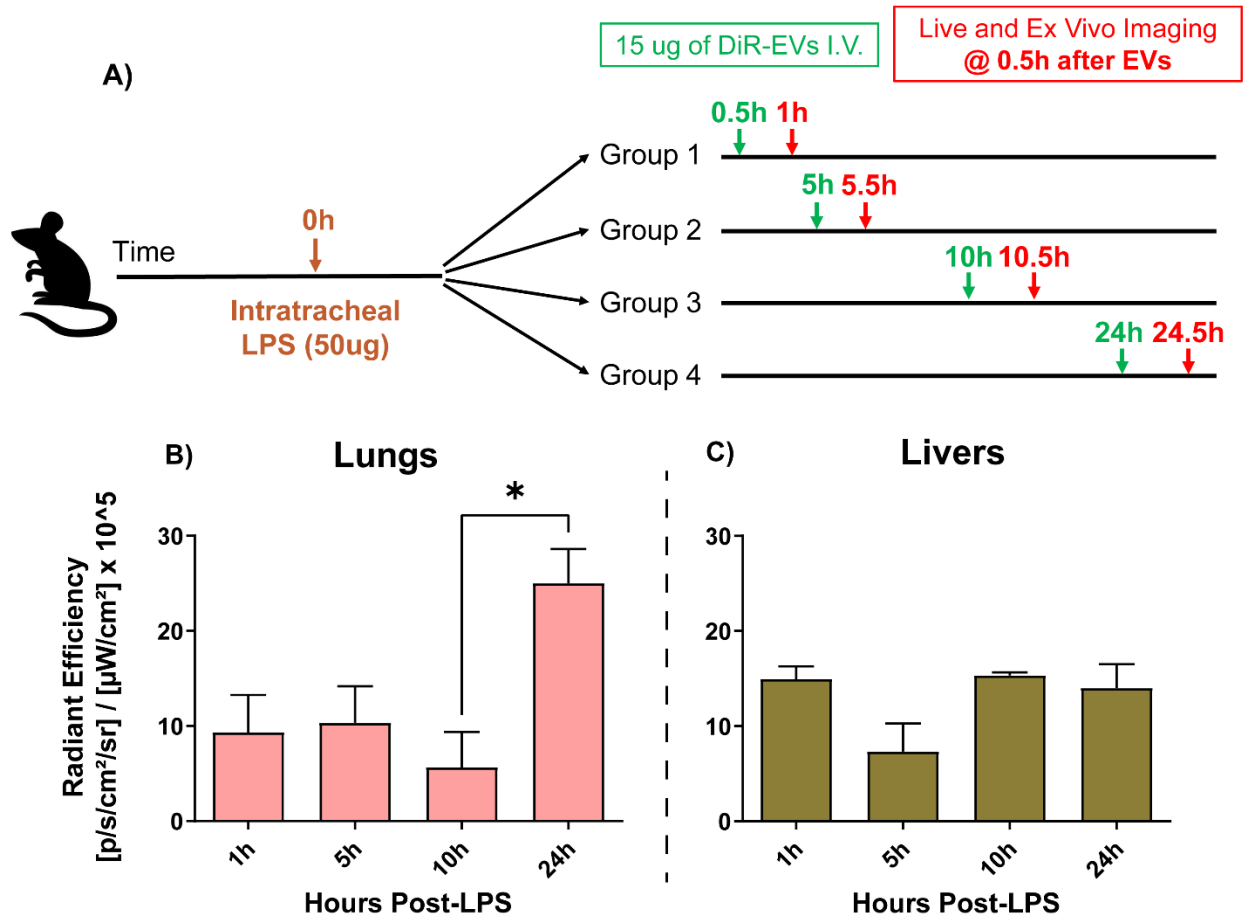


Figure 5.4. Optimal timing of MSC-EV administration.

MSC-EVs were labelled with DiR to evaluate the optimal time of administration during ALI. (A) Study design of the experiment including intratracheal delivery of DiR MSC-EVs (15μg) at 0.5h, 5h, 10h or 24h after disease induction, followed by live and *ex vivo* fluorescence imaging 0.5h after EV administration. (B) Lung and (C) liver fluorescence as measured by radiant efficiency. Optimal timing of EV administration for lung accumulation was found to be at 24h after disease induction. Data are presented as mean ± SEM, N=3 per group, * P<0.05.

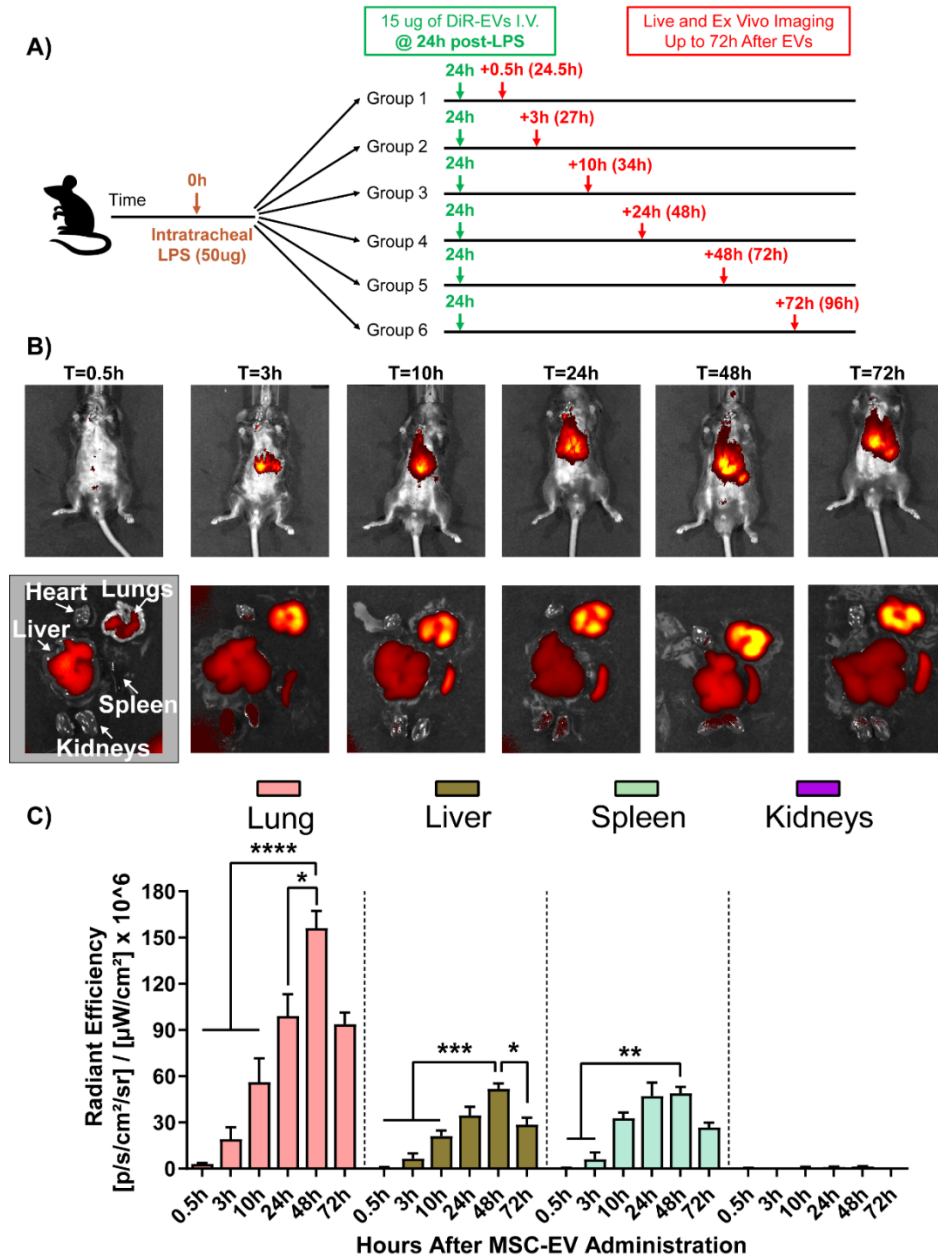


Figure 5.5. Biodistribution analysis of MSC-EVs during LPS-induced ALI.

(A) Study design in which DiR labelled MSC-EVs were administered at 24h after ALI induction, followed by fluorescence imaging at various times after EV delivery. (B) Representative live and *ex vivo* images are shown. (C) Comparison of radiant efficiency between the lungs, liver, spleen and kidneys. Distribution of MSC-EVs was greatest after 48h, and lung fluorescence was 3-fold higher than the spleen/liver and 80-fold higher than the kidneys at peak fluorescence. N=4-10 per group, data presented as mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

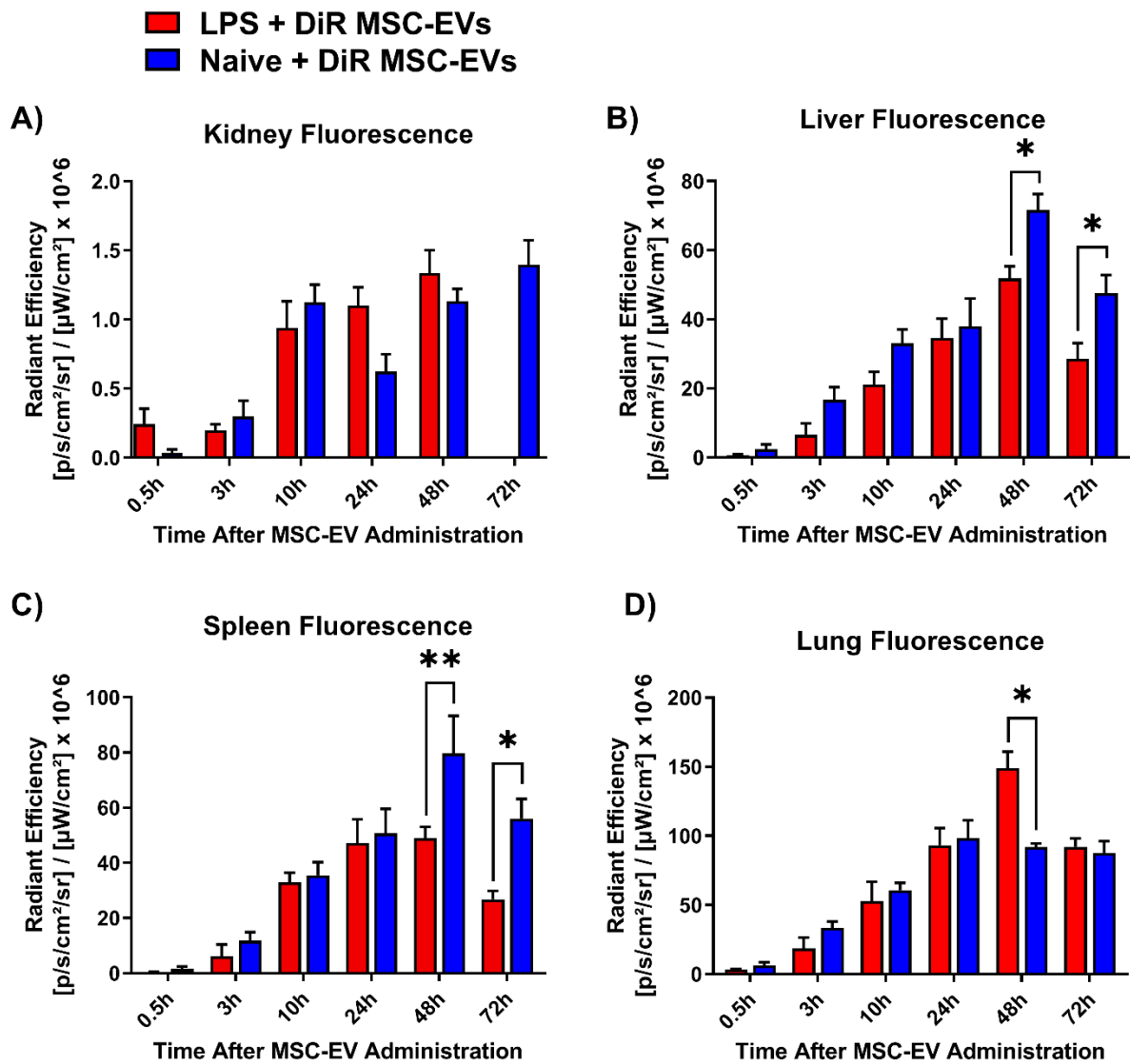


Figure 5.6. MSC-EV biodistribution between ALI and naive mice.

Comparison of DiR labelled MSC-EV biodistribution between ALI and naive mice. *Ex vivo* fluorescence in (A) kidneys, (B) liver, (C) spleen, and (D) lungs found significantly elevated pulmonary distribution during ALI, whereas greater accumulation to the liver and spleen were seen in naive mice. N=4-10 per group for ALI mice, N=3-6 for naive mice, data presented as mean ± SEM, * P<0.05, ** P<0.01.

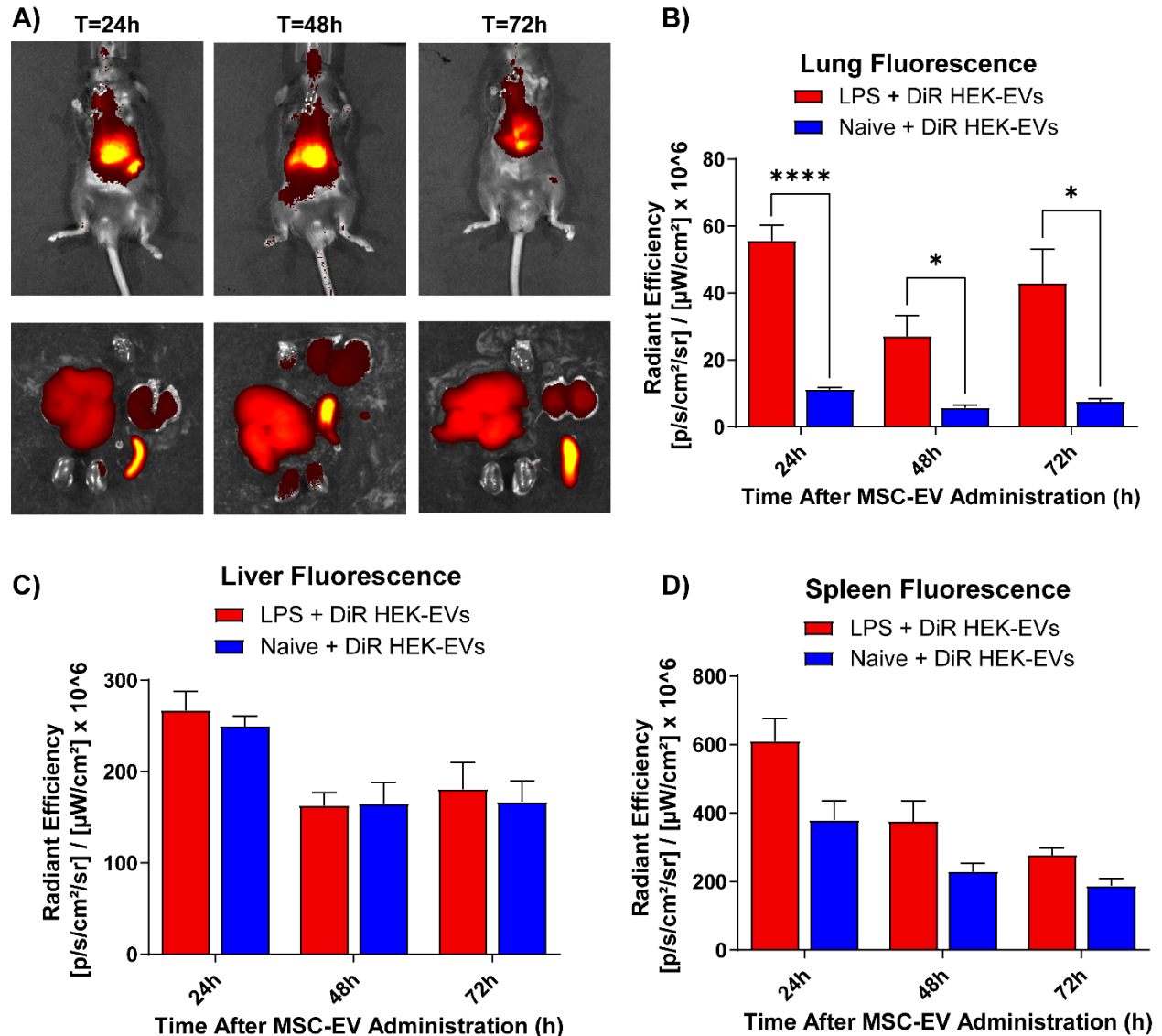


Figure 5.7. Biodistribution of DiR labelled EVs derived from HEK293T cells

(A) Representative live and *ex vivo* organ images at 24h, 48 and 72h after HEK-EV administration in ALI mice. Comparison of EV distribution between mice with LPS-induced ALI and naïve controls found significantly elevated (A) lung accumulation of EVs during ALI with no changes to (B) liver or (C) spleen. N=5-6 per group for ALI mice, N=3-6 for naïve mice, data presented as mean ± SEM, * P<0.05, **** P<0.0001.

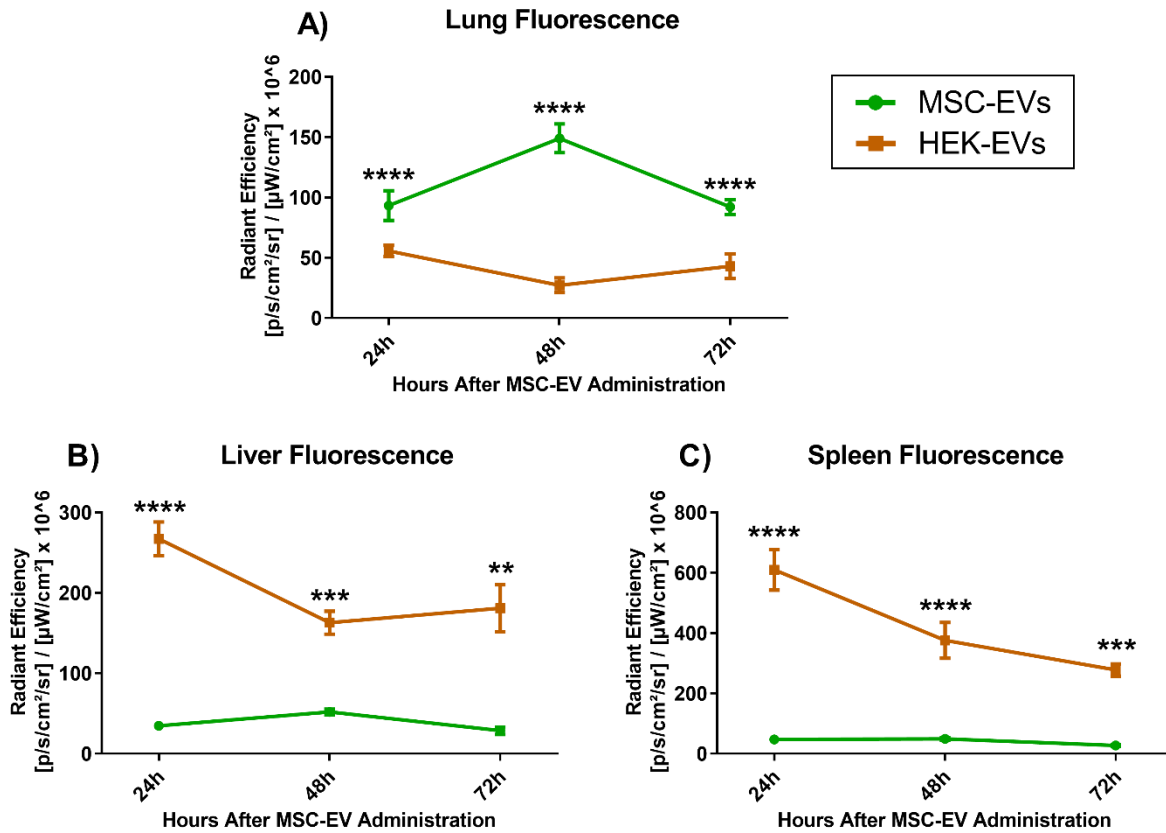


Figure 5.8. Biodistribution of DiR labelled EVs derived from MSCs and HEK293T cells during ALI.

(A) MSC-EVs demonstrated markedly elevated lung tropism as compared to HEK-EVs. In contrast, HEK-EVs exhibited greater distribution to the (B) liver and (C) spleen. N=4-10 per group for MSC-EVs, N=5-6 per group for HEK-EVs, data presented as mean ± SEM, ** P<0.01, *** P<0.001, **** P<0.0001.

Chapter 6 – Arming the Vesicular Stomatitis Virus with SARS-CoV-2 Spike Protein to Create a Model of Severe COVID-19 Acute Respiratory Distress Syndrome

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Author Contributions:

AT: Devised research question, developed experimental design, conducted the animal studies, and analyzed the data. Wrote the manuscript and generated the tables/figures.

DC and CL: Research technicians. Contributed to designing/conducting experiments and revision of manuscript

TJ and CI: Provided the SARS-CoV2 pseudovirus for experiments. Contributed to manuscript writing and revisions.

DJS and MML: Principal investigators of project. Contributed to designing experiments and revision of manuscript.

6.1 Abstract

INTRODUCTION:

Acute respiratory distress syndrome (ARDS) is the leading cause of mortality in COVID-19. An accessible preclinical model would greatly facilitate our understanding of COVID-19 ARDS and the development of novel therapies. However, current models using live SARS-CoV2 are limited by costly Biosafety Level 3 facilities which are not widely available. This study aimed to create an animal model of COVID-19 ARDS that is inexpensive, broadly accessible and less restricted by biohazard requirements.

METHODS:

We developed a pseudotyped version of vesiculovirus bearing the SARS-CoV2 spike protein (VSV-CoV2-S). Wildtype and K18 human ACE2 (K18-hACE2) transgenic mice were inoculated with intratracheal VSV-CoV2-S to induce acute lung injury (ALI). Disease severity score (0-30, combination of murine grimace scale and sepsis score), temperature and weight were recorded daily. Plasma, lung tissue and bronchoalveolar lavage fluid (BALF) were analyzed for clinically relevant features of ALI: (1) vascular permeability, (2) inflammation, and (3) histopathological injury (0-1). Data are reported as mean $\pm$ SEM.

RESULTS:

A dose ranging study (1E5-2.4E7 pfu) determined that 1E7 and 2.4E7 pfu of VSV-CoV2-S induced clinical, molecular and histological evidence of ALI in K18-hACE2 mice. To ascertain if ALI required hACE2 expression, we inoculated transgenic or wildtype mice with VSV-CoV2-S (1E7 pfu). In K18-hACE2 mice, there was a marked increase in disease severity score (17.0 $\pm$ 1.2; P<0.001) associated with significant declines in weight/temperature (~25%, P<0.001) and 100% mortality by day 6. Examination of BALF from K18-hACE2 mice inoculated with VSV-CoV2-S

revealed immune cell infiltration, high inflammatory cytokine levels and increased protein content consistent with lung vascular hyperpermeability ($P<0.01$). In contrast, all wildtype mice survived until day 10 with no clinical or molecular evidence of ALI. Histological lung injury score was markedly increased in K18-hACE2 mice (0.73 ± 0.04 , $P<0.001$), with findings of diffuse alveolar damage, pneumocyte hyperplasia, pulmonary hemorrhage and microthrombi formation. In contrast, wildtype mice receiving VSV-CoV2-S showed healthy lung tissue architecture, indicating that hACE2 expression is necessary for our pseudovirus to induce lethal lung injury.

CONCLUSIONS:

Our findings indicate that intratracheal delivery of VSV-CoV2-S caused severe ALI/ARDS in K18-hACE2 transgenic but not wildtype mice, which mirrored the reported preclinical effects of live SARS-CoV2. Transgenic mice inoculated with our pseudovirus also exhibited similar histopathological features to those observed in patients with COVID-19 ARDS. Importantly, our novel mouse model can be used safely in standard Biosafety Level 2 facilities to study COVID-19 ARDS, thereby enabling more widespread research into its pathogenesis and the development of novel therapies.

6.2 Introduction

Since the first reports of COVID-19 from Wuhan, China in December 2019, there have been nearly 550 million confirmed cases and over 6 million deaths worldwide.⁷⁷ Although many COVID-19 patients will recover with favorable outcomes, 5% of non-immunized infected individuals developed severe disease including acute respiratory distress syndrome (ARDS) at the beginning of the pandemic.^{84,91} In patients hospitalized due to the original SARS-CoV2 infections, COVID-19 associated ARDS accounted for 20 to 70% of admitted patients,^{99,469,470} and 100% of those requiring mechanical ventilation.⁴⁷¹ Moreover, ARDS remains to be the leading cause of death in COVID-19.⁸⁹ Unfortunately, survivors of ARDS face weeks of supportive therapy in the intensive care unit and long-term morbidity.

Central to the pathophysiology of COVID-19 is the binding of the SARS-CoV2 spike glycoprotein to angiotensin-converting enzyme 2 (ACE2) receptors on the surface of lung epithelial cells.¹¹³ Initial infection of the nasal cavity in the upper respiratory tract may produce a more mild, localized infection. However, SARS-CoV2 replication in the distal pulmonary airway and alveolar epithelium results in extensive inflammation.¹¹⁸ During disease progression, infected cells, alveolar macrophages and recruited leukocytes secrete a myriad of pro-inflammatory cytokines (e.g. IL-6, IL-1 β , TNF α , IFN γ) that induce diffuse pulmonary injury and lung vascular hyperpermeability.¹²⁰ Exudation of proteinaceous debris, fluid and blood into the interstitium and alveolar lumen leads to alveolar collapse and impairs gas exchange. In COVID-19 associated ARDS, many patients present with hypercoagulable states and exhibit endothelial dysfunction and *in situ* thrombosis.¹⁰⁵⁻¹⁰⁷ Although there were initial speculations that COVID-19 ARDS may differ from classical ARDS requiring alternative approaches for disease management,^{472,473} this has not been borne out by subsequent experiments that have shown typical features of ARDS present

during severe SARS-CoV2 infections.^{103,104,474} Despite these insights, it is clear more robust evidence and preclinical models are essential to understand the pathogenesis for COVID-19 associated ARDS.

An accessible and inexpensive preclinical animal model that reproduces the pathophysiological manifestations by which SARS-CoV2 induces infection would greatly accelerate the development of novel therapies and vaccines for COVID-19 related ARDS. Mouse models have many advantages because of their affordability and well-defined genetic backgrounds, as well as the availability of transgenic animals expressing human ACE2 receptor for binding of SARS-CoV2; however, working with the live SARS-CoV2 requires cumbersome and costly Biosafety Level 3 facilities, which are not widely available around the world. While the administration of recombinant spike protein can mimic some of the features of COVID-19 ARDS, it is costly to produce and requires adjuvant insults (e.g. intratracheal acid) in order to produce severe lung injury and therefore, representing a pathogenesis that is quite dissimilar to the human clinical disease.¹¹¹

In this study, we generated a pseudotyped version of a replication-competent vesicular stomatitis virus (VSV), a commonly used oncolytic virus with a favourable clinical safety profile,^{475,476} to express the SARS-CoV2 spike protein on its surface (named “VSV-CoV2-S”). By selecting VSV as our viral backbone, we were able to study its effects using Biosafety Level 2 facilities as VSV is well known to cause subclinical infections in humans with minimal evidence of person to person transmission.⁴⁷⁷ We show that intratracheal inoculation of VSV-CoV2-S in transgenic mice expressing human ACE2 induced severe ALI, faithfully reproducing many features characteristic of COVID-19 associated ARDS. In contrast, wildtype mice receiving VSV-CoV2-S exhibited normal pulmonary architecture with no clinical or molecular signs of disease,

indicating that an interaction with human ACE2 was necessary for the pseudovirus to induce lethal lung injury. These results mirror the published effects of live SARS-CoV2 without the restraints of needing Biosafety Level 3 facilities. Importantly, the pulmonary infection produced by our VSV-CoV2-S pseudovirus represents a novel and broadly applicable model of COVID-19 related ARDS. This model may be well suited for studies addressing the pathogenic mechanisms of SARS-CoV2 and/or for the development of novel therapies.

6.3 Materials and Methods

6.3.1 Study Approval and Reporting

Animal study protocol (OHRI3352) was approved by the Animal Care Committee of the University of Ottawa. The Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines 2.0 were applied for reporting of our animal experiments.⁴⁷⁸

6.3.2 SARS-CoV2 Pseudotyped Virus Production

Vero E6 cells were seeded in 1700 cm³ roller bottles (Corning) at approximately 5E7 cells per bottle in 200 mL Dulbecco's Modified Eagle Medium (DMEM, supplemented with 10% FBS and 25mM HEPES) and incubated at 37°C for 2 days. Cells were infected with VSV-Spike at a multiplicity of infection of 0.05 and incubated at 34°C for approximately 48 hours (or until ~90% cytopathic effect was evident). Supernatants were pre-cleared by centrifuging at 1500 rpm for 15 min at 4°C (Beckman Coulter, Allegra X-14R benchtop centrifuge). Pre-cleared supernatants were then filtered using a 0.22µm polyethersulfone filter (500mL Stericup polyethersulfone filter system; EMD Millipore) and the virus was concentrated by centrifugation at 12,000 rpm for 2 hours at 4°C (JLA 16.250 rotor; Beckman Coulter Avanti JE centrifuge). Concentrated virus was resuspended in cold phosphate buffered saline (PBS, 300µL/roller bottle) and stored at -80°C.

6.3.3 VSV Titer By Plaque Assay

Viral titers were determined by serial dilution plaque assays. Vero E6 cells were plated in 6-well plates (6E5 cells per well) and incubated overnight at 37°C. A 10-fold serial dilution for each virus was completed by adding virus stock to serum-free DMEM in a total volume of 1 mL per dilution. Media was removed from the wells and 500 µL of dilutions 10^{-3} to 10^{-8} were added to each well. After 45 minutes of incubation at 37°C (with rocking every 15 minutes), inoculum was removed by aspirating and replaced with a 1:1 mixture of 1% agarose and DMEM (2X concentrated with 20% FBS) overlay and incubated at 34°C. After 48 hours of incubation at 34°C, plates were fixed in a 3:1 mixture of methanol:acetic acid for 1 hour at room temperature. Overlay was removed and plates were washed gently with water followed by staining with crystal violet (0.1% crystal violet in 80% MeOH in water) for approximately 20 minutes at room temperature. After staining, plates were washed twice with water and left to dry overnight. Viral titers were calculated using the following equation with plaque counts (plaque forming units, pfu) from wells containing 10 to 100 plaques: Titer (pfu/mL) = No. of plaques / dilution factor * volume (mL).

6.3.4 Mice

Male hemizygous transgenic mice (8-10 weeks, 20-25g) expressing the hACE2 receptor under the human keratin 18 (K18) promoter were obtained from the Jackson Laboratory (Stock No.: 034860, B6.Cg-Tg(K18-ACE2)2Prlmn/J) (Bar Harbor, ME, USA).¹³⁹ Wildtype (non-carrier) C57BL/6 mice were obtained from the same colony and used as controls in animal experiments. In total, n=35 K18-hACE2 transgenic mice were used for all experiments. Specifically, n=20 K18-hACE2 mice were used for dose-response studies. N=15 K18-hACE2 mice and n=15 wildtype mice were used in experiments comparing severity of VSV-CoV2-S induced ALI.

6.3.5 Mouse Genotyping

The genotypes of wild-type and hemizygous K18-hACE2 mice were determined by PCR. Tail snips were collected from mice and processed with the REDExtract-N-AMP Tissue PCR Kit (Sigma-Aldrich, Missouri, USA) as per the manufacturer's instructions to isolate genomic DNA. Briefly, tail snips were incubated in a 4:1 mix of Extraction Solution and Tissue Preparation Solution for 10 minutes at room temperature, followed by samples being heated to 95°C for 3 minutes. Heating was followed by addition of Neutralization Solution B to each sample. The PCR was run using the isolated genomic DNA, the REDExtract-N-Amp™ PCR Reaction Mix, and a 1.3µM mix of the following primers: Transgene Forward: 5' - GAC CCC TGA GGG TTT CAT ATA G – 3', Wild Type Forward: 5' - AAG TTG GAG AAG ATG CTG AAA GA – 3', Reverse (Common): 5' - CAC CAA CAC AGT TTC CCA AC – 3'. PCR cycling parameters were as follows: initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation at 95°C for 30s, annealing at 62°C for 1 minute, and extension at 72°C for 1 minute. Cycling was followed by extension at 72°C for 2 minutes with amplicons electrophoresed in a 1.2% agarose gel.

6.3.6 Lung Infection with VSV-CoV2-S

Mice were anesthetized with intraperitoneal injection of 120/10 mg of ketamine/xylazine per kg, orally intubated with a sterile 22-gauge catheter, and administered intratracheal VSV-CoV2-S resuspended in 50µL of PBS. For studies with multiple experimental groups, mice were randomized using a computer-generated randomizer on Microsoft Excel (Microsoft, Washington, USA). All investigators were blinded to mice allocation during the experimental progress and outcome assessment. All animal experiments, observational outcomes and tissue collection were performed in a Biosafety Level 2 facility at the University of Ottawa according to the recommendations by Animal Care and Veterinary Services for safe laboratory handling of VSV.

6.3.7 Animal Observational Outcomes

Observational outcomes including weight, temperature, disease severity score and survival of mice were recorded at baseline, 6h after infection and monitored daily. Weight and temperature are reported as absolute values and percent change from baseline. Internal temperature was assessed through a rectal probe thermometer. Disease status of mice were quantified using a “Disease Severity Score” that combines the Murine Sepsis Score⁴⁷⁹ and Murine Grimace Scale⁴⁸⁰ to feature 8 variables: (1) Fur Appearance, (2) Level of Consciousness, (3) Activity, (4) Response to Stimulus, (5) Eye Appearance, (6) Respiration Rate, (7) Respiration Quality and (8) Facial Grimace Score. All variables were scored from 0-4 with exception of facial grimace score (0-2). A cumulative score of 21 or higher indicated impending endpoint. This composite scoring system was justified based on our recent Delphi consensus study of ALI surrogate humane endpoints.⁴⁸¹ Further details regarding scoring of each variable can be found in **Supplemental File D1**. *A priori* parameters for mice endpoint included weight loss >20% from baseline, internal temperature <34°C, and signs of distress through disease severity score or observation (e.g. severe respiratory distress, piloerection, pronounced hunched posture and dehydration).

6.3.8 Measurements of BALF Cell Count, Protein Concentration and Cytokine Analysis

Blood was collected through the inferior vena cava, centrifuged at 2000g and the plasma was removed and subsequently stored at -80°C. The right lung was lavaged three times with 500µL of saline to collect bronchoalveolar lavage fluid (BALF) for outcome analysis. BALF cell concentration was determined using a CellDropTM automated cell counter (DeNovix, DE, USA). BALF was then centrifuged at 800g to pellet cells, while the supernatant was retained and frozen at -80°C until future analysis.

Lung vascular permeability was assessed through BALF protein concentration measured using the DC protein assay (Bio-Rad, ON, Canada). IL1-β and IL-6 concentrations in BALF and

plasma were measured using murine cytokine-specific ELISA kits (Thermo Fisher Scientific, MA, USA).

6.3.9 Lung Histology

Two separate midsections of the left lung were used for histopathological analysis. Lung tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sliced into 5 µm thick sections with a microtome (Leica Microsystems, ON, Canada). Lung sections were stained with hematoxylin and eosin. Lung section images were acquired by PANNORAMIC DESK scanscope (3DHISTECH, Hungary) and analysed using CaseViewer (3DHISTECH, Hungary). Five random high-power fields (40x magnification) for each mouse were analyzed for histological lung injury. Scoring was conducted in a randomized and blinded fashion as per the American Thoracic Society guidelines.^{57,69} In brief, five parameters were considered including: (1) Intra-alveolar cell infiltration, (2) cell infiltration into interstitial space, (3) hyaline membranes, (4) proteinaceous debris in airspace (e.g. fibrin strands), and (5) alveolar septal thickening. An average lung injury score per mouse was calculated with a potential maximum score of 1.

6.3.10 Statistical Analysis

Data are represented as mean ± SEM unless otherwise stated. Statistical analysis was completed using Student's t-test, One-Way ANOVA (>2 treatment groups) or Two-Way ANOVA (if two categorical variables, e.g. time and dose) followed by Tukey's post-hoc test for multiple comparisons. All statistical analyses were conducted using GraphPad Prism (version 9.1.0 for Windows; GraphPad Software). P-value < 0.05 was considered significant.

6.4 Results

6.4.1 Dose-Response Analysis of VSV-CoV2-S in K18-hACE2 Transgenic Mice

A dose escalating study was conducted to determine the number of plaque forming units (pfu's) of VSV-CoV2-S pseudovirus necessary to produce an ALI in K18-hACE2 transgenic mice. Mice were randomized to receive a single intratracheal injection of VSV-CoV2-S at 1E5, 1E6, 1E7 or 2.35E7 pfu (maximum feasible dose), or PBS as vehicle control. Animals were monitored for wellness, weight and internal temperature at the timepoints shown in **Figure 6.1A**, with a planned end-study at 5 days. One mouse died peri-procedurally (intent to treat with 1E6 pfu) and one mouse received only 1E7 pfu as opposed to the target dose 2.35E7pfu due to limitations in virus supply. This mouse was subsequently analyzed as a subject in the 1E7 pfu experimental group.

6.4.2 Dose Response Analysis – Physiological Outcomes

There was a transient increase in disease severity scores in both PBS and virus inoculated mice at 6h post inoculation, likely due to anesthesia and procedure (**Figure 6.1B**). All mice normalized to baseline wellness by 48 hours. By 5 days post inoculation, K18-hACE2 transgenic mice receiving the two highest viral doses exhibited a second increase in the disease severity score ($P<0.05$) as compared to all other pseudovirus doses and control mice (2.35E7 pfu, score of 5.5 ± 0.9 ; and 1E7 pfu, score of 4.2 ± 1.4 ; versus 0-1 for other doses) (**Figure 6.1B**). Similarly, the two highest VSV-CoV2-S doses resulted in a marked decline to weight ($P<0.05$) ($-8.1\pm1.5\%$ for 2.35E7 pfu and $-9.2\pm2.1\%$ for 1E7 pfu versus 0.0 to -1.5% for other doses) and a trend for reduced temperatures ($-7.4\pm1.8\%$ for 2.35E7 pfu and $-9.0\pm1.0\%$ for 1E7 pfu versus 0.0 to -0.9% for other doses, not statistically significant) (**Figures 6.1C and 1D**).

6.4.3 Dose Response Analysis – Cellular and Molecular Outcomes

In K18-hACE2 transgenic mice receiving 2.35E7 pfu and 1E7 pfu doses of VSV-CoV2-S, lung vascular permeability was increased as BALF protein levels were 3.0 ± 0.3 mg/mL and 3.1 ± 0.1

mg/mL, respectively, which were 8-fold greater ($P<0.001$) than mice receiving PBS or $1\text{E}5$ pfu (**Figure 6.2A**). Total cell concentration in BALF was $2.6\pm0.7 \times 10^6$ cells/mL ($2.35\text{E}7$ pfu VSV-CoV2-S) and $3.7\pm1.0 \times 10^6$ cells/mL ($1\text{E}7$ pfu VSV-CoV2-S), which were 4-9 fold and 6-13 fold higher than other treatment groups, respectively (**Figure 6.2B**).

IL-6, a primary mediator of pulmonary inflammation in COVID-19 associated ARDS, was measured in both BALF and plasma of mice. Normal baseline levels of IL-6 were found in BALF of mice receiving PBS, $1\text{E}5$ pfu or $1\text{E}6$ pfu of VSV-CoV2-S. While IL-6 concentrations were elevated in mice inoculated with $1\text{E}7$ pfu (336.6 ± 143.5 pg/mL) and $2.35\text{E}7$ pfu (484.2 ± 266.3 pg/mL) (**Figure 6.2C**), these findings were not significant due to high variability and low number of animals. Plasma concentrations of IL-6 were negligible across all experimental groups (**Figure 6.2D**), consistent with a localized lung injury response within the first 5 days after VSV-CoV2-S infection.

6.4.4 Dose Response Analysis – Histopathological Lung Injury

Histological lung sections were examined and compared for findings of VSV-CoV2-S induced ALI. In K18-hACE2 transgenic mice treated with PBS, no signs of ALI were observed. Furthermore, lungs of mice receiving $1\text{E}5$ pfu or $1\text{E}6$ pfu of pseudovirus were also found to have healthy alveolar structure with no indications of pulmonary inflammation or mononuclear cell infiltration (**Figure 6.3**). In contrast, mice that received $1\text{E}7$ pfu or $2.35\text{E}7$ pfu of VSV-CoV2-S exhibited severe interstitial pneumonia with diffuse alveolar damage (**Figure 6.3**). Lung sections revealed interstitial, peribronchial and perivascular infiltration by mononuclear cells with areas of hyperplastic type II pneumocytes and alveolar hemorrhage. Lung injury scores in K18-hACE2 transgenic mice receiving $1\text{E}7$ pfu (0.67 ± 0.10) or $2.35\text{E}7$ pfu (0.72 ± 0.17) of VSV-CoV2-S were 3-to 4-fold higher than mice receiving the lower viral doses (**Figure 6.3**). Together, these results

suggested that while there was a delayed ALI response from the two highest pseudovirus doses, longer follow up was needed to assess peak severity.

6.4.5 Intratracheal Inoculation of VSV-CoV2-S Induces a Fatal ALI Response in K18-hACE2 Transgenic Mice

Based on the dose-response analysis, $1\text{E}7$ pfu was found to be the lowest dose of VSV-CoV2-S to result in lung injury by day 5 in K18-hACE2 transgenic mice. Next, we explored the effects of lung VSV-CoV2-S on ALI features and survival at later timepoints, and the dependence of the ALI response on binding of VSV-CoV2-S to hACE2. VSV-CoV2-S was administered to both K18-hACE2 transgenic and wildtype mice with predefined end-studies at days 5, 7 and 10 with tissue collection for comparative analyses (**Figure 6.4A**). One mouse died peri-procedurally on day 0, and one mouse did not receive any pseudovirus.

At days 5 and 6 post VSV-CoV2-S inoculation, K18-hACE2 transgenic mice exhibited a significant ($P<0.0001$) increase in disease severity score (3.9 ± 1.0 for day 5, 17.0 ± 1.2 for day 6) (**Figure 6.4B**), which were associated with declines to both weight ($-14.3\pm1.4\%$ for day 5, $-25.2\pm0.9\%$ for day 6, **Figure 6.4C**) and temperature ($-8.9\pm4.9\%$ for day 5, $-27.3\pm12.9\%$ for day 6, **Figure 6.4D**) ($P<0.0001$). In contrast, wildtype animals showed no evidence of lung injury or systemic infection as their disease severity score, weight, and temperature were all unchanged compared to baseline.

Given the substantial deterioration in wellness scores, the remaining K18-hACE2 transgenic mice were humanely euthanized on day 6. Survival analysis demonstrated 100% mortality for K18-hACE2 transgenic mice by day 6 after receiving intratracheal $1\text{E}7$ pfu of VSV-CoV2-S, while all wildtype mice survived to day 10 and remained healthy (**Figure 6.4E**). These

findings are consistent with the requirement of hACE2 receptor expression for VSV-CoV2-S to cause lethal lung injury.

6.4.6 Molecular and Histological Outcomes Confirm Severe ALI Response in K18-hACE2 Transgenic Mice

Lung Cell Infiltration and Vascular Permeability

Total cell concentration in BALF was used as a measure of cell infiltration into lung airspaces. After VSV-CoV2-S inoculation, BALF cell concentration was nearly 4-fold higher in K18-hACE2 transgenic mice as compared to wildtype mice on both day 5 ($p<0.05$) and day 6 ($p<0.01$) (**Figures 6.5A**). Moreover, BALF protein concentration in K18-hACE2 transgenic mice on day 5 and day 6 were 9-fold ($P<0.001$) and 8-fold ($P<0.01$) higher than wildtype mice, respectively (**Figures 6.5C**). Both BALF cell concentration (**Figure 6.5B**) and protein levels (**Figure 6.5D**) did not differ between K18-hACE2 transgenic mice euthanized on days 5 and 6. These findings suggest that intratracheal delivery of VSV-CoV2-S causes significant lung vascular injury and hyperpermeability that is dependent on interaction with the hACE2 receptor.

Histopathological Lung Injury

Histological assessment of whole lung sections from wildtype mice suggested no pulmonary injury at day 6 after VSV-CoV2-S inoculation. (**Figure 6.6A**). In contrast, K18-hACE2 transgenic mice exhibited widespread lung inflammation and injury (**Figure 6.6B**). At higher power magnification, wildtype mice showed uniformly thin and normal appearance of alveolar walls (**Figure 6.6C**), which is consistent with healthy lung structure at all timepoints post-inoculation despite receiving a high dose of $1E7$ pfu VSV-CoV2-S. In contrast, lung sections of K18-hACE2 transgenic mice showed evidence of diffuse alveolar damage, alveolar septal

thickening and interstitial pneumonia with type II pneumocyte hyperplasia and pulmonary hemorrhage (**Figure 6.6D**). Thrombus formation was evident (**Figure 6.6E and F**) along with signs of vasculitis (**Figure 6.6G**), which are all features described in severe COVID-19 associated ARDS. Perivascular, peribronchial and interstitial infiltration by mononuclear cells including histiocytes, lymphocytes and alveolar macrophages were frequently observed (**Figure 6.6G**). In some mice, bronchial epithelial cells were swollen, degenerated, and desquamated with cytoplasmic blebbing (**Figure 6.6H**). Lastly, pseudovirus inoculation resulted in production of protein exudates (e.g. fibrin strands) and deposition of hyaline membranes, hallmark features of clinical ARDS (**Figure 6.6H**).⁵⁷ These findings are in agreement with a severe ALI phenotype and was consistently observed across all K18-hACE2 transgenic mice treated with 1E7 pfu of VSV-CoV2-S.

A standardized lung injury score was derived from stained lung sections. On day 5, K18-hACE2 transgenic mice were found to have a lung injury score 4.3-fold higher than wildtype mice (0.73 ± 0.04 vs. 0.17 ± 0.01) ($P < 0.0001$) (**Figure 6.7A**). On day 6, the disparity in histopathological injury was even greater as lung injury score in K18-hACE2 transgenic mice (0.85 ± 0.02) was 6-fold higher than wildtype mice (0.14 ± 0.05) (**Figure 6.7A**). When comparing the lung injury scores in wildtype mice between days 5, 6 and 10, no differences were observed (**Figure 6.7B**). Conversely, K18-hACE2 mice euthanized on day 6 were found to have markedly elevated histological lung injury as compared to transgenic mice euthanized on day 5 ($P < 0.01$) (**Figure 6.7C**).

6.5 Discussion

Since the start of the pandemic, small animal models have been vital to the understanding of COVID-19 pathogenesis, therapeutic testing, and vaccine development. They serve as an affordable and efficient approach to explore the potential efficacy of novel therapeutics prior to performing expensive non-human primate studies and clinical trials. Given that SARS-CoV2 exhibits poor affinity to rodent ACE2, a number of studies have utilized various tactics to express human ACE2 in mouse lungs to enable SARS-CoV2 infection, including viral transduction,^{146,482-484} knockin models using CRISPR/Cas9,⁴⁸⁵ or a transgenic model with hACE2 expression associated with the lung epithelial HFH4/FOXJ1 promoter.¹⁴⁵ Unfortunately, many of these mouse models did not result in lethal lung injury from SARS-CoV2 inoculation.^{146,482,484,485} In comparison, K18-hACE2 transgenic mice created for the first SARS coronavirus pandemic has now become the most prevalent animal model to study COVID-19 due to its development of severe disease and clinically relevant histopathology.^{140-143,148} Importantly, published studies using SARS-CoV2 *in vivo* to date have required access to Biosafety Level 3 facilities, which represents a significant barrier for widespread participation in researching the pathobiology of COVID-19 ALI as well as the development of novel therapeutics. As well, many of these small animal studies introduced SARS-CoV2 by intranasal injection, resulting in severe neurological deficits from viral neurotropism that may have attributed to mortality.^{141,144} Intratracheal delivery is known to be a more effective and superior method for ALI induction due to broader lung distribution and distal lung involvement.⁴⁸⁶⁻⁴⁸⁸ For this reason, we chose to utilize intratracheal administration for our VSV-CoV2-S pseudovirus to ensure development of a relevant model of COVID-19 ARDS.

When 1E7 pfu of intratracheal VSV-CoV2-S was administered to wildtype mice, there were no signs of disease up to 10 days post inoculation. Conversely, all K18-hACE2 transgenic

mice met humane surrogate endpoints by day 6 after exhibiting severe deteriorations in weight, temperature and wellness. Considerable inflammatory cell infiltration and injury to the alveolar endothelial-epithelial barrier was induced by VSV-CoV2-S inoculation. Interestingly, our findings from VSV-CoV2-S delivery mirror the results of many studies that administered live SARS-CoV2 in K18-hACE2 transgenic mice. Firstly, both SARS-CoV2 and our VSV-CoV2-S display high specificity for hACE2 receptor binding as wildtype mice infected with either virus do not present any ALI features.^{114,143,144,150,485,489} Secondly, intratracheal delivery of VSV-CoV2-S resulted in physiological outcomes of lethal disease requiring euthanasia of K18-hACE2 mice by day 6, a timeline of progression that is nearly identical to that produced by 1E5 pfu of SARS-CoV2.^{140-144,490} Third, the severity of lung injury as assessed using standardized histological scoring was very similar to findings seen after SARS-CoV2 infection of K18-hACE2 transgenic mice. Hence, the pulmonary disease generated by intratracheal delivery of VSV-CoV2-S at 1E7 pfu may be comparable to 1E5 pfu of intranasal SARS-CoV2. Importantly, our VSV-CoV2-S pseudovirus can be safely handled in commonly accessible standard Biosafety Level 2 facilities and requires a 100-fold lower inoculation dose, therefore offering a more convenient animal model of COVID-19 related ALI for widespread uptake.

VSV-CoV2-S infection of K18-hACE2 transgenic mouse lungs also exhibit pathological features that closely resemble the human disease. In one of the first case reports of a patient who succumbed to COVID-19 associated ARDS, examination of biopsy samples suggested interstitial pneumonia with bilateral diffuse alveolar damage, desquamation of lung epithelium and hyaline membrane composition,⁴⁹¹ which were further confirmed in multiple post-mortem patient autopsies.⁴⁹² Moreover, a recent systematic review of 263 cases found SARS-CoV2 induced lung injury to be characterized by reactive pneumocyte hyperplasia, leukocytic infiltration of the

interstitium and extensive exudative/proliferative alveolitis,⁴⁹³ all of which were seen in K18-hACE2 transgenic mice after inoculation with our VSV-CoV2-S pseudovirus. Within the pulmonary vasculature, histological samples in patients and our mouse model featured capillary congestion, vasculitis and arteriolar thromboembolic lesions often associated with COVID-19 pneumonia.⁴⁹⁴ Overall, the similarities in histopathology suggest that VSV-CoV2-S induced ALI in mice may be a suitable model of severe COVID-19 associated ARDS. Future studies will compare differences in lung injury response with pseudotyped VSV bearing the spike protein of SARS-CoV2 variant strains, and evaluate the influence of sex, age and comorbidities in our mouse model.

6.6 Conclusions

In summary, our VSV-CoV2-S pseudotyped virus results in severe lung injury and inflammation after intratracheal delivery to K18-hACE2 transgenic mice. Molecular and histological findings of clinically relevant ALI features along with declining weight and temperatures mirror the reported effects of live SARS-CoV-2 administration in K18-hACE2 transgenic mice. However, we were able to conduct these studies in a standard Biosafety Level 2 facility, which are commonly found at research institutes around the world. Thus, we have established a new mouse model of COVID-19 associated ARDS that is broadly accessible, thereby enabling more widespread research into its pathogenesis and development of novel therapeutics and vaccines.

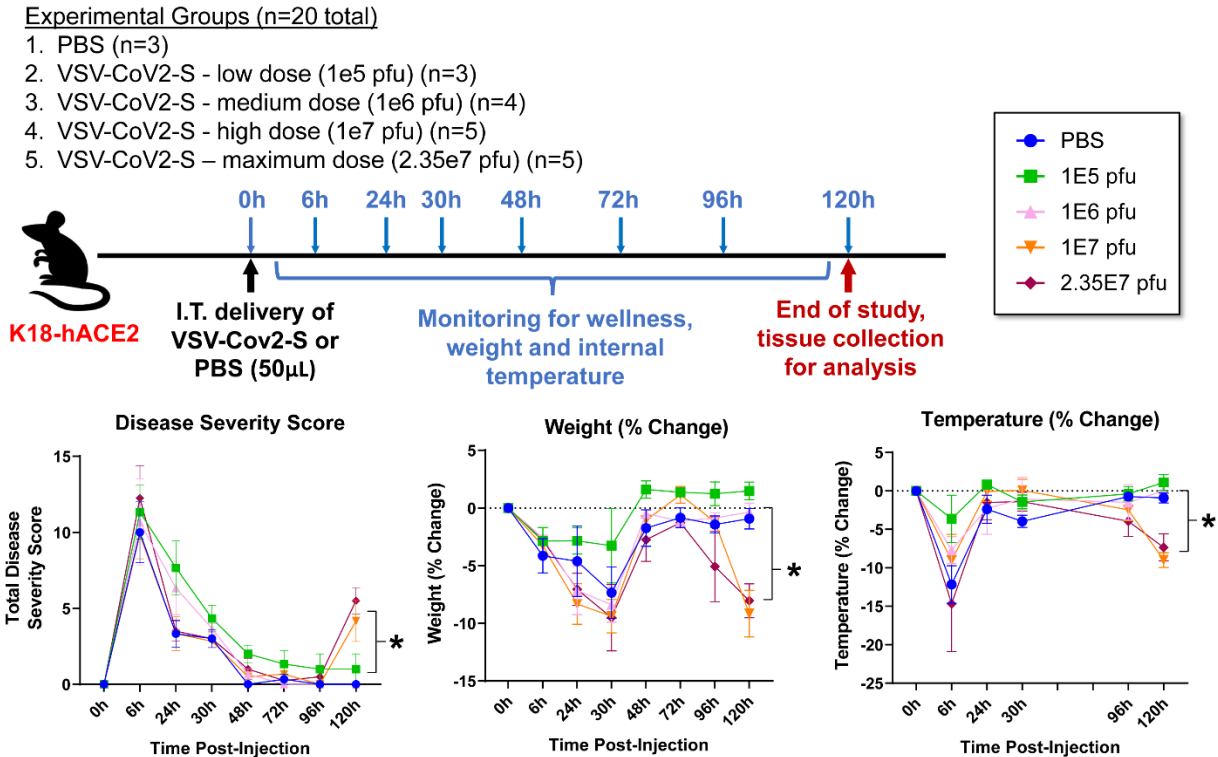


Figure 6.1. Observational outcomes from VSV-CoV2-S dose-response study in K18-hACE2 transgenic mice.

Observational outcomes in K18-hACE2 transgenic mice receiving 1E5, 1E6, 1E7 or 2.35E7 pfu of VSV-CoV2-S or PBS as control. (A) Study design for dose-response analysis of VSV-CoV2-S administration. (B) Disease severity score, (C) percent change in weight, and (D) percent change in temperature of K18-hACE2 transgenic mice up to 128 hours after intratracheal inoculation with VSV-CoV2-S. No data points for mice temperature were collected at 48h and 72h. N=3-6 per timepoint. Data are presented as mean±SEM, * P<0.05.

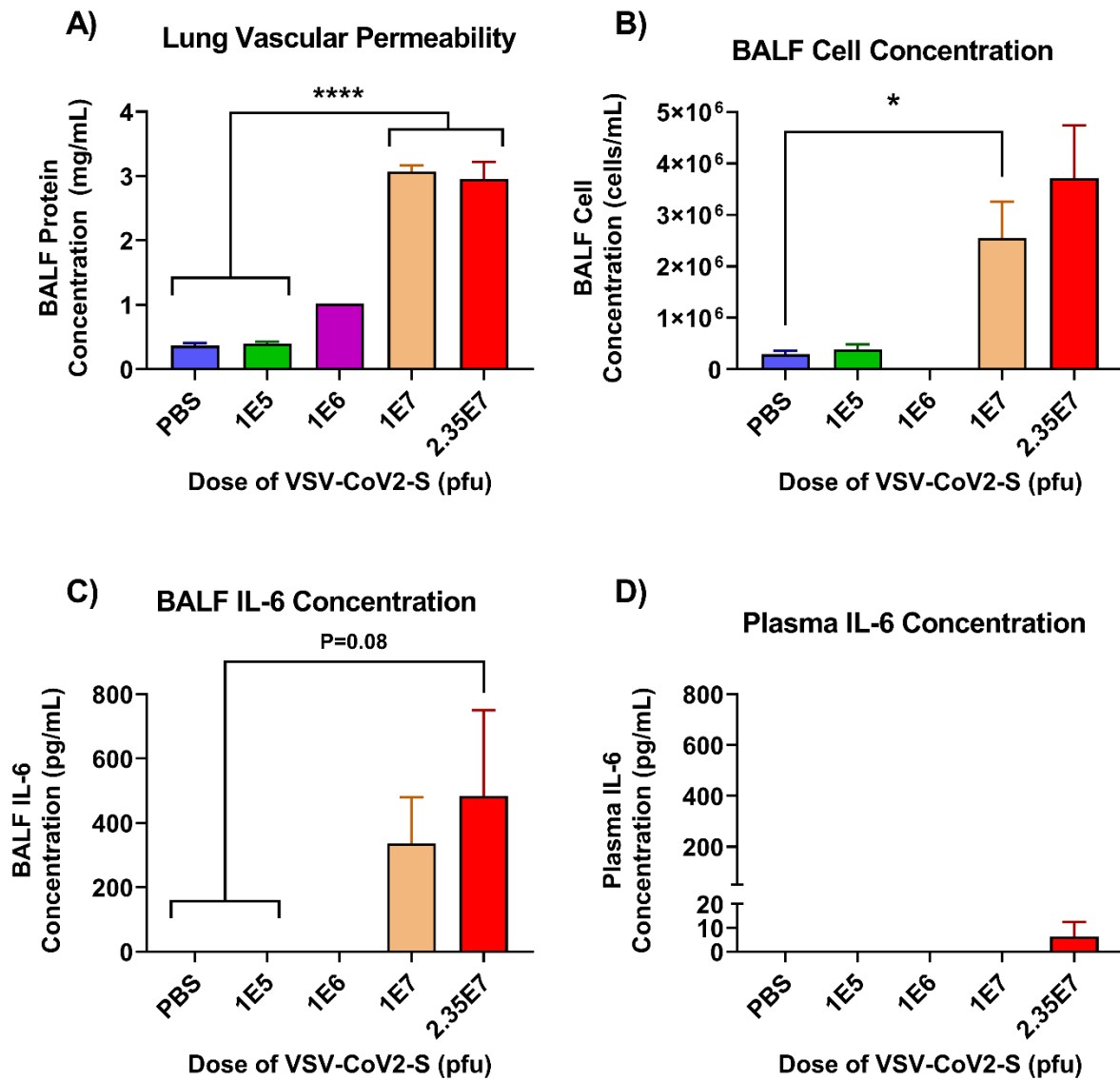


Figure 6.2. Molecular markers of ALI at 5 days post-inoculation in dose-response study of K18-hACE2 mice.

Molecular markers evaluated for evidence of ALI induction in K18-hACE2 transgenic mice at day 5 post-inoculation with 1E5, 1E6, 1E7 or 2.35E7 pfu of VSV-CoV2-S, or PBS as control. (A) Lung vascular permeability as measured by BALF protein concentration, (B) BALF cell concentration, (C) BALF IL-6 concentration, and (D) plasma IL-6 concentration. N=3-6 for BALF data in mice receiving PBS, 1E5 pfu, 1E7 pfu, or 2.35E7 pfu. N=1 for BALF data in mice receiving 1E6 pfu due to technical difficulties in BALF collection. N=3-4 for plasma IL-6 data. Data are presented as mean±SEM, * P<0.05, **** P<0.0001.

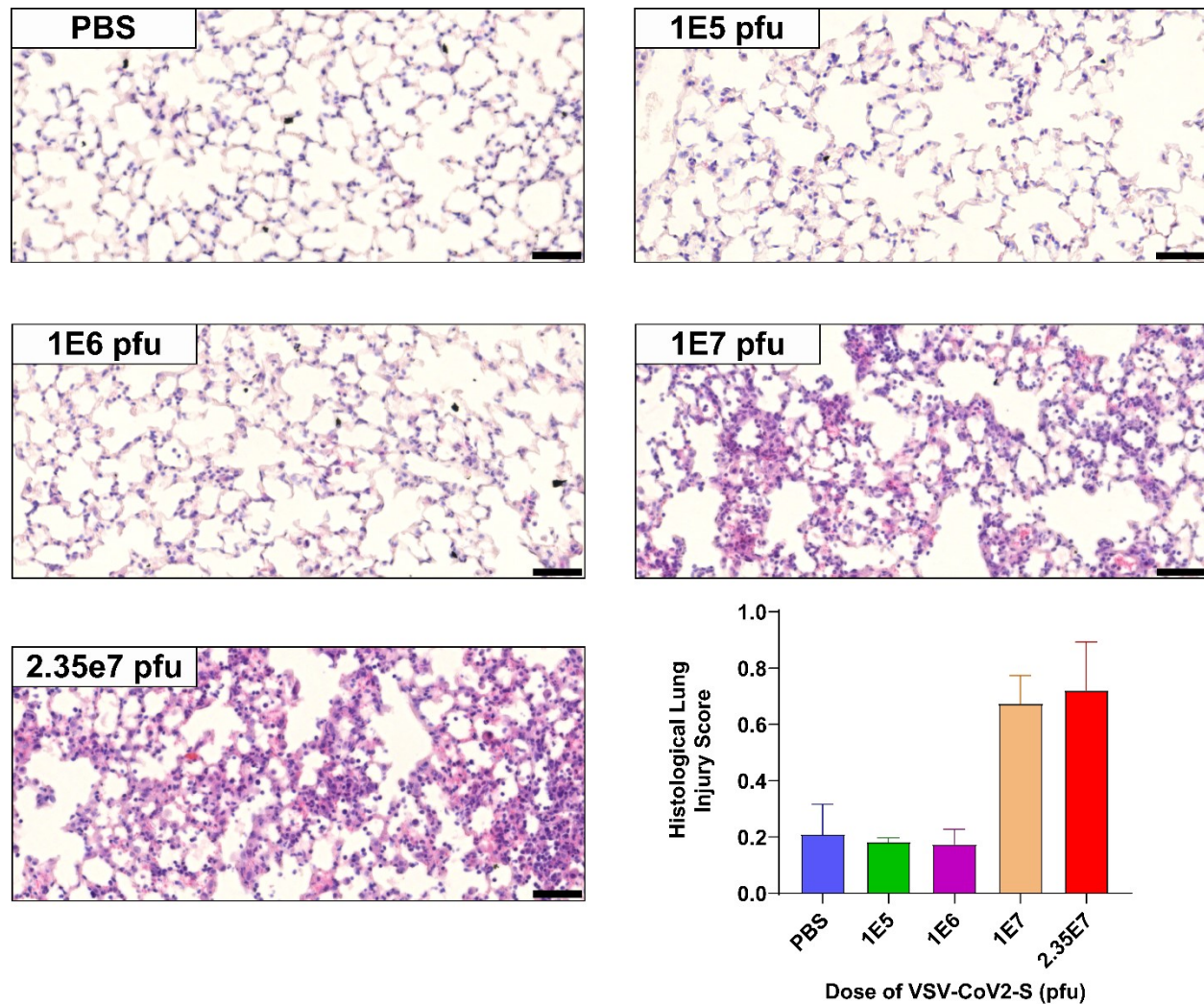


Figure 6.3. Lung histology at 5 days post-inoculation in dose-response study of K18-hACE2 mice.

Histological analysis of lung sections in K18-hACE2 transgenic mice at 5 days post-inoculation with 1E5, 1E6, 1E7 or 2.35E7 pfu of VSV-CoV2-S, or PBS as control. Representative histological images are shown for each dose of VSV-CoV2-S. Histological lung injury score was evaluated as per the American Thoracic Guidelines, which includes assessment for: (1) Intra-alveolar cell infiltration, (2) cell infiltration into interstitial space, (3) hyaline membranes, (4) proteinaceous debris in airspace (e.g. fibrin strands), and (5) alveolar septal thickening. N=2-5 per dose. Data are presented as mean±SEM with a maximum score of 1.0.

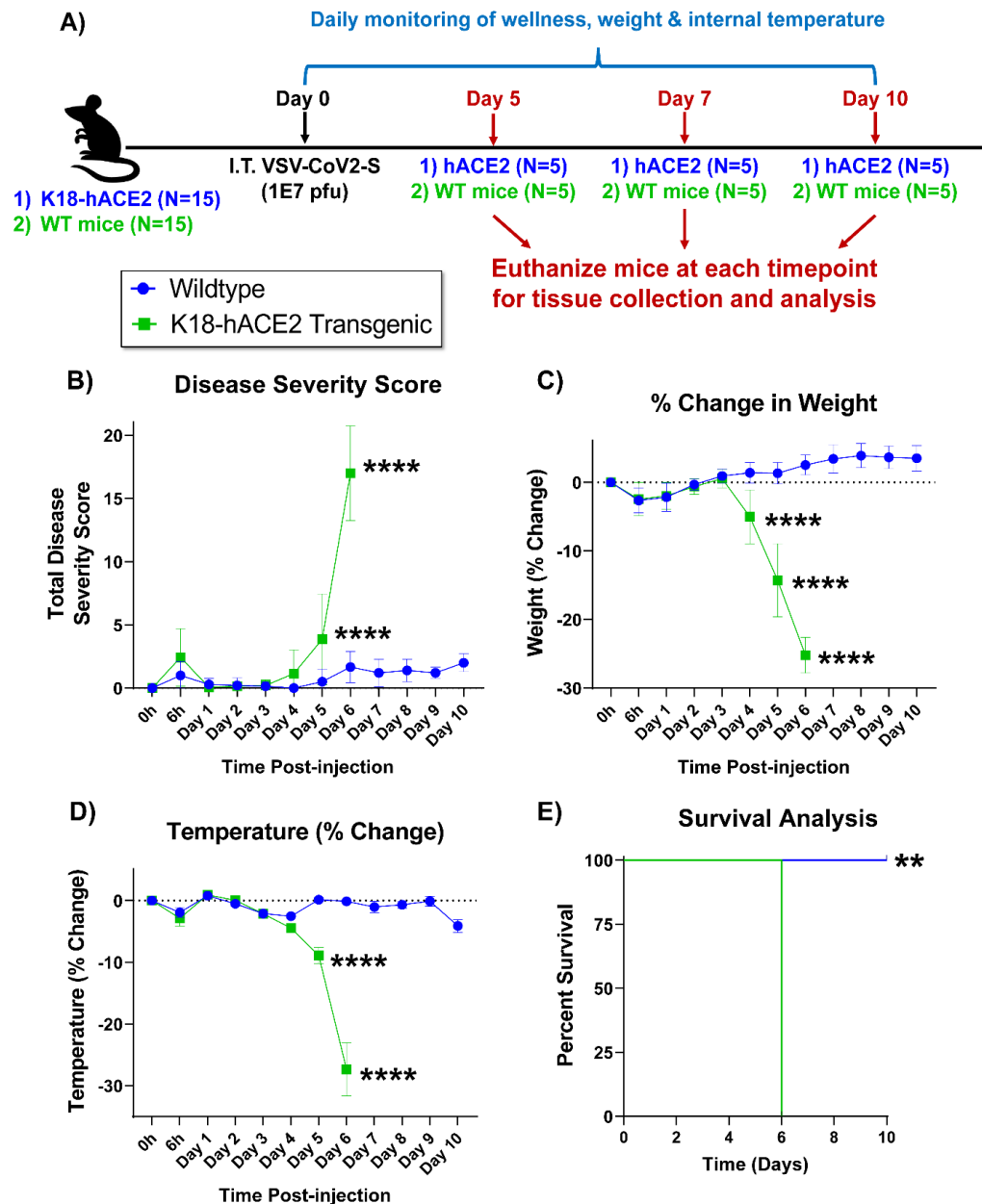


Figure 6.4. Comparison of observational outcomes between wildtype and K18-hACE2 mice.

Observational outcomes comparing administration of 1E7 pfu VSV-CoV2-S in wildtype (blue) and K18-hACE2 transgenic (green) mice. (A) Study design, (B) disease severity score, (C) percent change in weight, and (D) percent change in temperature (N=14 for days 1-5, N=9 for day 6, and N=5 for days 7-10). (E) Survival analysis up to 10 days post-inoculation of VSV-CoV2-S (N=5 per group). Data are presented as mean±SEM, ** P<0.01, **** P<0.0001.

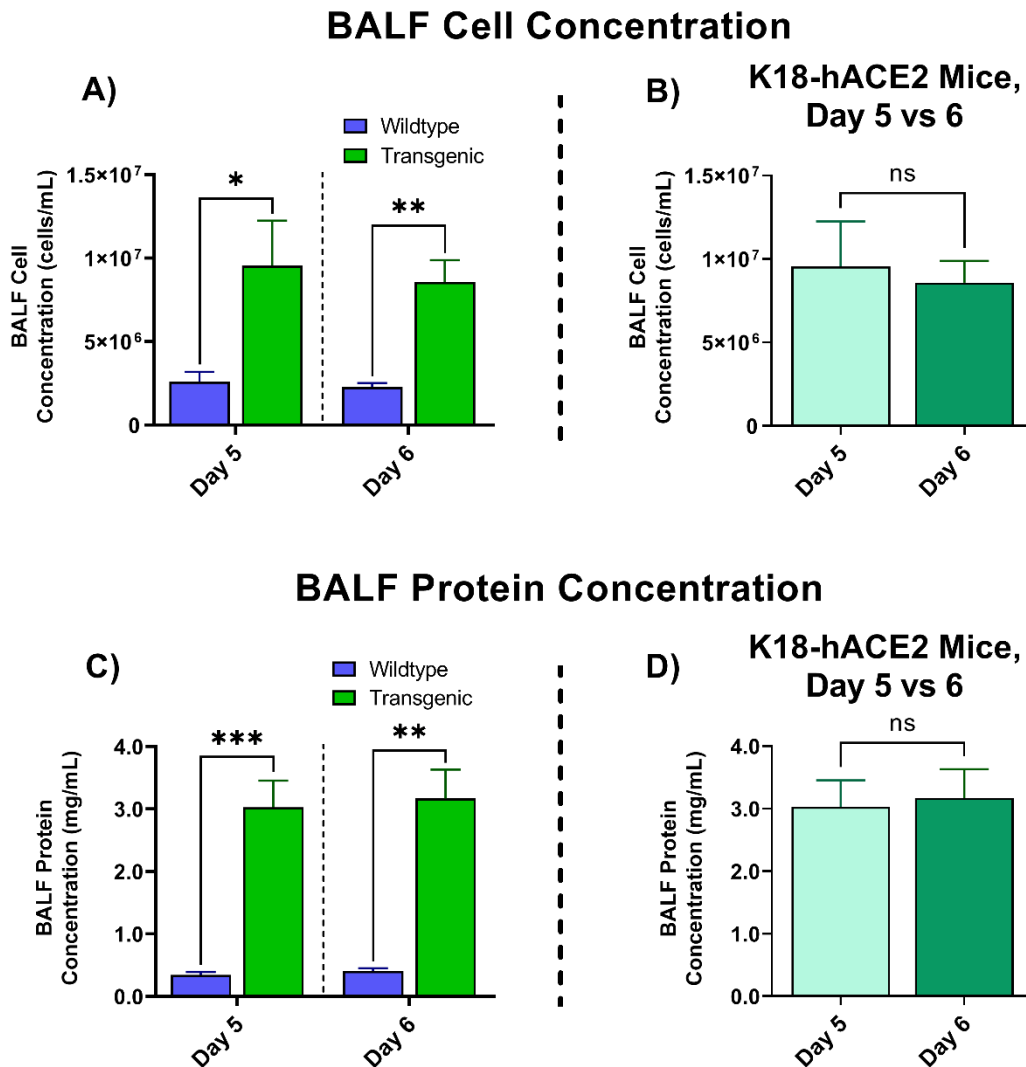
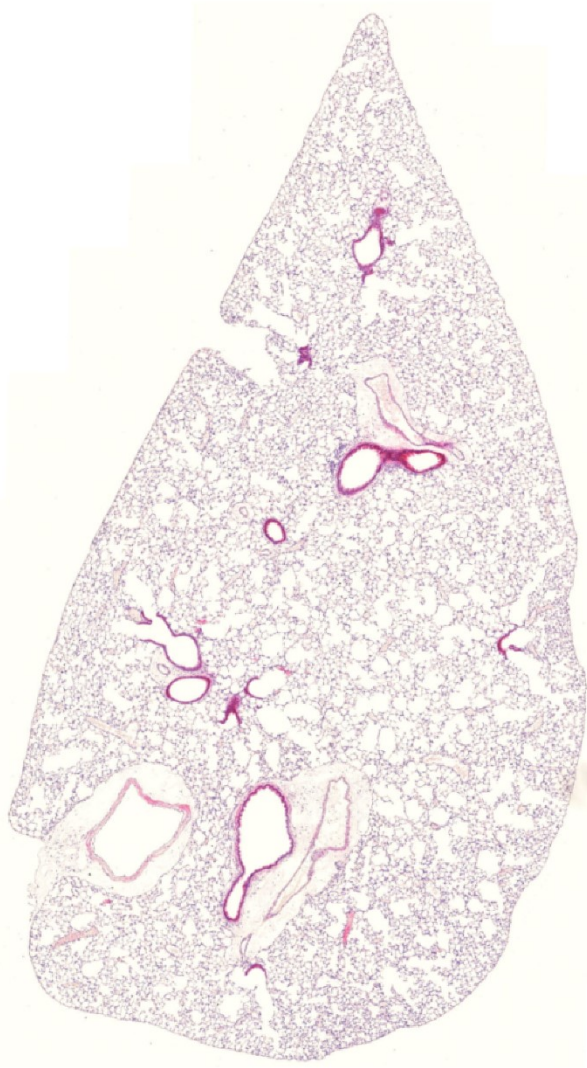


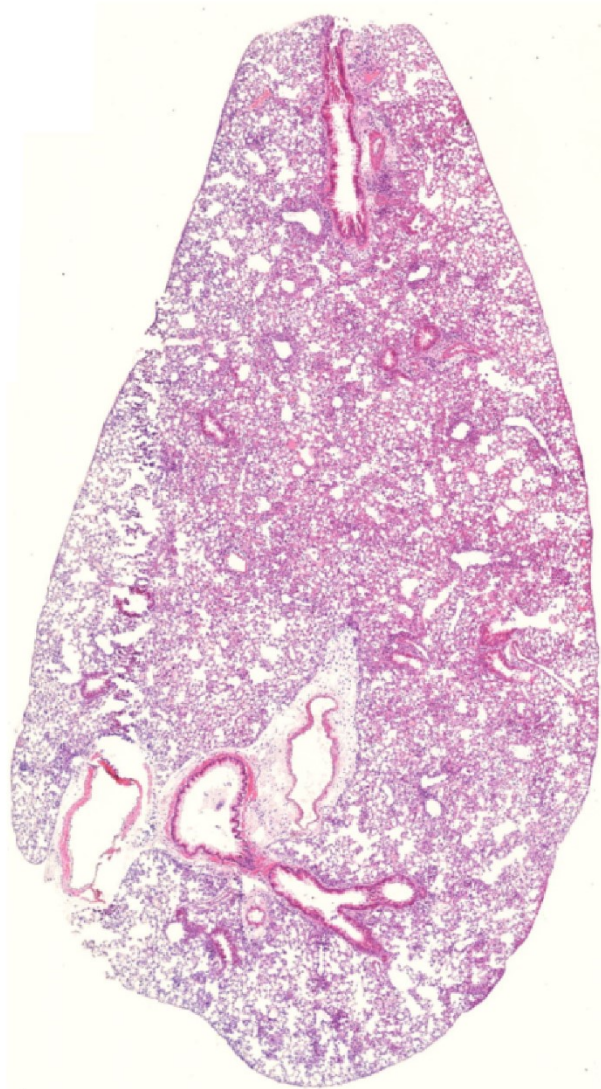
Figure 6.5. Comparison of alveolar cell infiltration and permeability between wildtype and K18-hACE2 transgenic mice.

Alterations to alveolar cell infiltration and pulmonary vascular permeability in wildtype (blue) and K18-hACE2 transgenic (green) mice from administration of 1E7 pfu VSV-CoV2-S. (A) K18-hACE2 mice exhibited augmented BALF cell concentration on days 5 (N=3-4 per group) and 6 (N=4-7 per group) as compared to wildtype mice. (B) In K18-hACE2 mice, no differences in BALF cell concentration were observed between days 5 (N=3) and 6 (N=7). (C) BALF protein concentration was significantly elevated in K18-hACE2 mice as compared to wildtype mice on days 5 (N=3-4 per group) and 6 (N=4-7 per group) post-inoculation. (D) No differences were observed when comparing BALF protein concentration in K18-hACE2 mice between days 5 (N=3) and 6 (N=7). Data are presented as mean±SEM, * P<0.05, ** P<0.01, *** P<0.001.

A) Wildtype



B) K18-hACE2



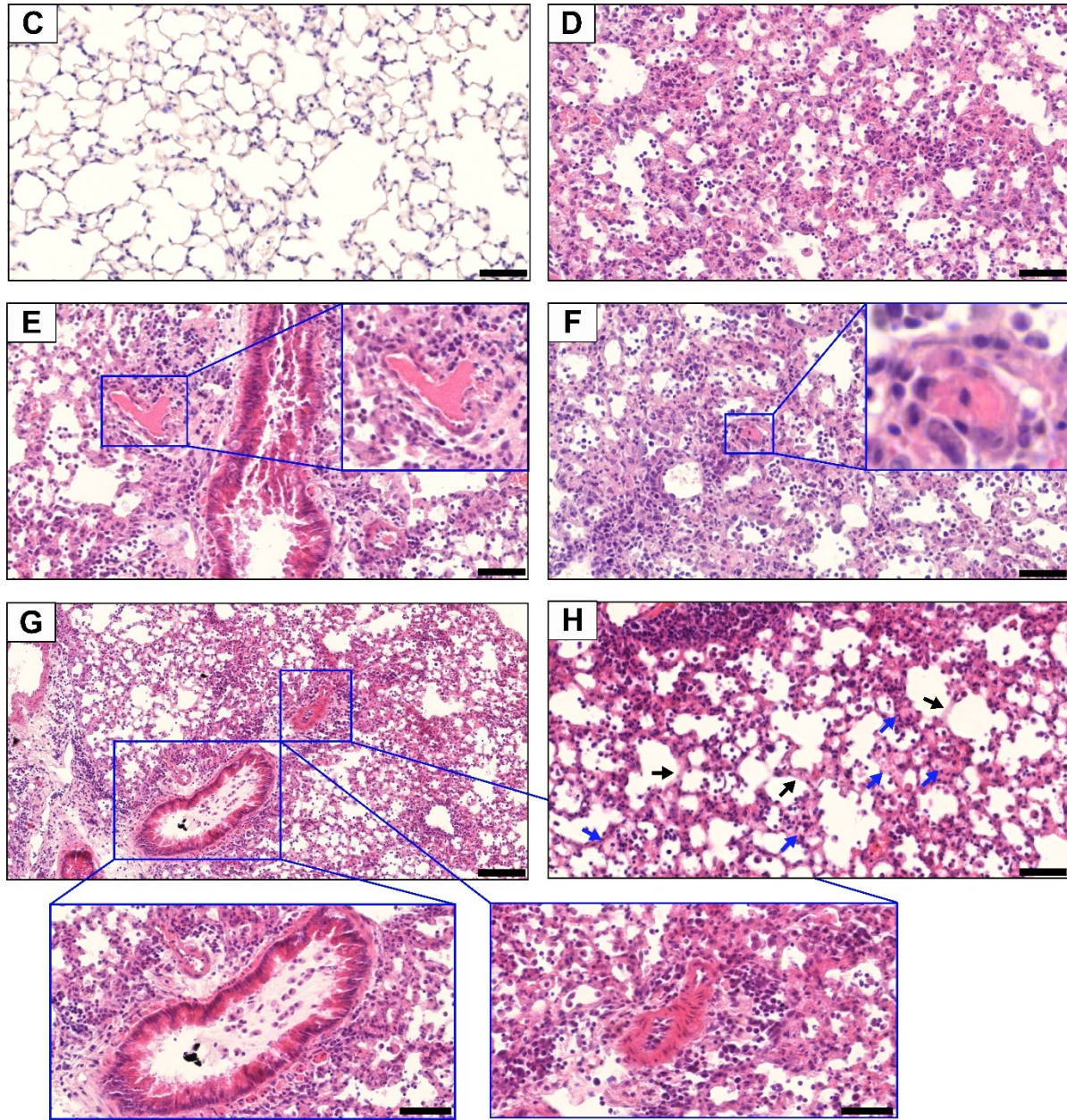


Figure 6.6. Representative histopathological images of lung sections in mice receiving VSV-CoV2-S pseudotyped virus.

Gross lung sections suggest severe lung injury and inflammation induced by 1E7 pfu of VSV-CoV2-S in (B) transgenic mice, but not (A) wildtype mice. (C) Microscopic images of lung sections in wildtype mice demonstrate healthy pulmonary structure with thin alveolar septum and normal distribution of alveolar macrophages and pneumocytes. Panels D–H are histological images obtained from lung sections of K18-hACE2 mice. (D) Diffuse alveolar damage, alveolar septal thickening, pulmonary hemorrhage, and interstitial/alveolar inflammatory cell infiltration. In (E) and (F), thrombi formation (blue box), perivascular inflammation and bronchial epithelial

cell desquamation is observed. (G) Peribronchial and intra-bronchiolar inflammatory cell infiltration with swelling, desquamation and degeneration of bronchial epithelial cells (left blue box). Perivascular cell infiltration alongside vasculitis (right blue box). (H) Hyaline membrane formation (black arrows) and fibrin deposition (blue arrows) are evident. Scale represents 50 μ m.

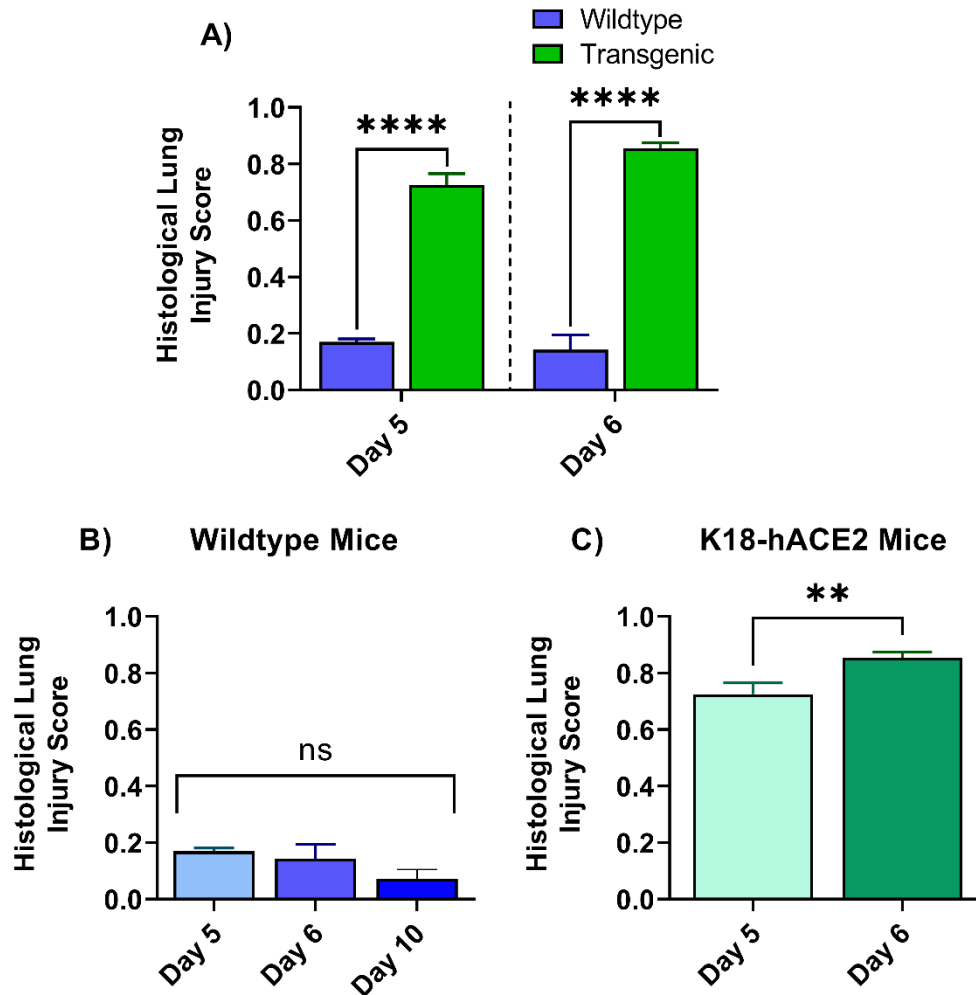


Figure 6.7. Comparison of histological lung injury score between wildtype and K18-hACE2 mice.

Histological lung injury score in wildtype (blue) and K18-hACE2 transgenic mice at 5- and 6-days post-inoculation with 1E7 pfu VSV-CoV2-S. (A) K18-hACE2 transgenic mice exhibited significantly worse histological lung injury scores at days 5 (N=4-5 per group) and 6 (N=4-9 per group). (B) Within wildtype mice, no differences in histological lung injury were observed between mice euthanized on days 5, 6 or 10 (N=4-5 per group). (C) K18-hACE2 transgenic mice at 6 days post-inoculation (N=9) demonstrated more severe lung injury than those euthanized on day 5 (N=4). Data are presented as mean±SEM with a maximum score of 1.0. ** P<0.01, **** P<0.0001.

Chapter 7 – General Discussion and Perspective

7.1 Summary

The investigations in Chapters 2 through 5 comprise four independent, but related studies that combined meta-research methodology with basic science techniques to develop a more thorough understanding of the optimal approaches for isolating and administering MSC-EVs. The aims of my research project were to: (1) synthesize data on the current experimental approaches, modifications and general efficacy of MSC-EVs in preclinical animal studies, (2) assess the *in vivo* efficacy of MSC-EVs to treat ALI and other pulmonary diseases, and (3) explore the biodistribution and kinetics of MSC-EVs during ALI as a prerequisite to designing optimal therapeutic strategies. Chapter 6 represents a supplemental investigation that arose in response to the SARS-CoV2 pandemic. This study aimed to establish a novel and more widely accessible animal model of COVID-19 associated ARDS. Moving forward, this model would be able to be utilized in evaluating the efficacy of MSC-EVs for COVID-19.

7.2 Evidence Map of Methodology, Study Design and Outcomes in Animal Studies of MSC-EVs

Over the past decade, there has been a rapid rise in interest for developing EVs as a cell-free therapy. Our understanding of EV biology has advanced considerably, which has resulted in meaningful changes to EV nomenclature, methodology and international guidelines outlining salient considerations for isolating and characterizing EVs. In preclinical interventional studies, the efficacy of MSC-EVs is often attributed to their bioactive cargo including microRNAs, proteins, and mRNAs. This mechanism by which EVs function has promoted the concept of optimizing therapeutic outcomes by engineering EVs to express specific molecular mediators. However, the extent of augmented efficacy by EV modifications was poorly understood.

In Chapters 2 and 3 of this thesis, we synthesized data from 206 animal studies of MSC-EVs and found nearly all studies reported significant improvements in at least one outcome. We further characterized studies by EV modifications. In articles that intended to enhance efficacy by preconditioning or altering packaged content within MSC-EVs, 70% of studies exhibited marked improvements to outcomes, organ function and survival from the delivery of modified EVs. We provided readers with a centralized index of critical microRNAs and proteins, and the most commonly targeted bioactive factors regulated by MSC-EVs. Hence, researchers can preferentially select a molecular target of interest, study the impact of its overexpression, and potentially augment the protective properties of MSC-EVs for clinical translation.

Importantly, our systematic review identified several methodological concerns. Despite detailed position statements regarding methodology from the ISEV, there is still no consensus on the most effective approaches. Accordingly, we reported considerable heterogeneity in EV terminology, experimental techniques for isolation and characterization, interventional traits and treatment protocols. There remains to be a large paucity of data concerning EV biodistribution and whether therapeutic effects of MSC-EVs are dose-dependent. Greater implementation of the MISEV2018 guidelines and attention to the issues identified in our systematic review may help accelerate the translational success of EV therapies.

Future Considerations

Taken together, our findings from Chapters 2 and 3 identified important, unanswered questions in the field of MSC-EV therapeutics that guided our focus in the remaining chapters.

- 1) Is there a single molecular mediator or mechanism that is critical for MSC-EV function, or is synergy between multiple factors necessary for efficacy?

- 2) Does the therapeutic efficacy of EVs vary depending on tissue source of MSCs (e.g., bone marrow, umbilical cord, adipose tissue), EV subtype (e.g. small vs medium/large EVs), donor characteristics (e.g., sex, age, health), and/or disease context (acute or chronic pathology)?
- 3) Is there one modality for EV isolation that enables more effective scale-up and therapeutic benefit?
- 4) How can we implement and improve adherence to international MISEV2018 guidelines to ensure standardization of EV nomenclature, characterization and isolation?

7.3 Therapeutic Efficacy of MSC-EVs for Respiratory Diseases

In Chapter 4, we followed up our broad systematic review in Chapter 3 with a meta-analysis to quantitatively evaluate the potency of MSC-EVs as therapy for acute and chronic pulmonary diseases. To also address the knowledge gaps identified from Chapter 3, we conducted subgroup analyses with aims of determining EV characteristics crucial to therapeutic efficacy. Administration of MSC-EVs led to marked improvements to nearly all clinically significant outcomes in animal models of ALI, bronchopulmonary dysplasia and pulmonary arterial hypertension. For all diseases, the various tissue sources of MSCs and routes of EV delivery were similar in their ability to generate therapeutic benefit. Interestingly, subgroup analysis indicates potentially differential effects in EV subtypes whereby small EVs were shown to be more consistently effective than large EVs. When evaluating studies by isolation technique, EVs enriched by ultracentrifugation led to greater benefits. However, these findings are from limited studies and more direct comparisons with equivalent starting material are warranted.

Similar to our findings in Chapter 3, there was still considerable inconsistencies in experimental approach, EV terminology and adherence to the MISEV 2018 international guidelines. Moreover, we found that the quality of reporting study design parameters was consistently incomplete across all studies, which was accompanied by an unclear risk of bias in experimental rigor. These findings are concerning as they hinder adequate interstudy comparisons to draw more robust conclusions on the most efficacious characteristics of MSC-EVs.

Notably, our group has also completed multiple systematic reviews and meta-analyses to further characterize the protective ability of MSC-EVs as an intervention for other disease states, including graft versus host disease,⁴⁹⁵ diabetic wounds,⁴⁴⁸ malignancy⁴⁴⁹ and myocardial infarction (manuscript in preparation).

Future Studies:

- 1) **Direct Comparisons to Discern the Most Important EV Characteristics:** Although we showed that there are distinct differences in benefit depending on EV subclass or enrichment technique, studies included for comparison may have had confounding variables associated with study design, animal models, dosage regimen, or interventional traits. Hence, there is a need for validation studies with head-to-head assessments of EV subtypes and/or isolation methods while ensuring all other variables are equivalent.
- 2) **Sensitivity Analysis by Risk of Bias:** Given the consistently unclear risk of bias in study design, the validity in findings from the included articles may not be as robust. As rigor and transparency in EV preclinical research is improved, data extracted from studies of low risk of bias may be given more weight to determine a more accurate estimate for the efficacy of MSC-EVs.

- 3) **Living Systematic Review:** Despite conducting an updated search, there were still a limited number of studies included for each domain of respiratory disease. A living systematic review can actively and dynamically search and incorporate new evidence from the most recent publications. Hence, this will provide greater confidence not only in our pooled estimates for overall efficacy of MSC-EVs, but also findings of differences due to EV characteristics.

7.4 Biodistribution of MSC-EVs During ALI

Our meta-analysis of MSC-EVs for respiratory conditions found only two studies that qualitatively observed EV distribution to the lungs after administration. No studies have provided quantitative analysis of MSC-EV kinetics or biodistribution during ALI. In Chapter 5, we demonstrated that systemic delivery of MSC-EVs at 24 hours after ALI induction results in greatest accumulation to the lung tissue. Interestingly, EVs derived from human umbilical cord MSCs displayed high lung tropism, which is further enhanced when administered as a rescue treatment during ALI. Our findings of preferential distribution of MSC-EVs to the lungs combined with the robust evidence of therapeutic efficacy from our meta-analysis in Chapter 4 highlight MSC-EVs as a promising cell-free intervention for the treatment of ARDS.

Future Studies:

- 1) **Comparing MSC Tissue Sources for Differences in Lung Tropism and Efficacy:**

Our study used umbilical cord tissue as the parent source of MSC-EVs given the non-invasive requirements for tissue collection, superior EV production and improved proliferative capacity. However, bone marrow MSCs remain to be the most common source for MSC-EV enrichment. Hence, future studies can investigate whether bone

marrow MSC-EVs exhibit similar lung tropism. Moreover, EVs isolated from both tissue sources can also be directly compared for their protective effects during ALI.

- 2) **Identifying and Manipulating the Molecular Mediators for Lung Tropism:** Our finding of preferential lung accumulation by MSC-EVs begs the question of what molecular factors may be involved in mediating this distribution pattern. To identify potential candidates responsible for this effect, future studies can apply proteomic analysis comparing the expression of various cell-membrane and cell adhesion molecules between MSC-EVs and HEK293T-EVs. The expression of target proteins can then be manipulated (e.g. downregulated or overexpressed) within MSC-EVs to evaluate their impact on lung biodistribution. If a molecular target can be identified, this may enable a mechanism to augment lung-specific targeting, thereby expanding and enhancing the benefits of EVs to pulmonary diseases beyond ALI.

7.5 A New Model of COVID-19 Associated ARDS

With restrictions implemented on routine, non-COVID lab work, Chapter 6 was an additional objective that utilized my experience with preclinical modeling of ARDS in combination with Dr. Carolina Ilkow's expertise in viral engineering. To better understand the pathophysiology of COVID-19 ARDS, scientists require a relevant preclinical model. Current models have multiple limitations: (1) working with SARS-CoV2 requires restrictive Biosafety Level 3 facilities, (2) SARS-CoV2 spike protein does not bind rodent ACE2, and (3) large animals are expensive with only mild respiratory manifestations. In Chapter 6, we successfully established a mouse model of severe COVID-19 associated ARDS using a VSV-CoV2-S pseudovirus, thereby enabling experiments to be performed within standard Biosafety Level 2 facilities. Importantly,

VSV-CoV2-S demonstrated high specificity for human ACE2 receptor binding that induces molecular and histopathological changes similar to SARS-CoV2 infection of transgenic mice and humans. These findings will hopefully allow more researchers around the world to investigate the pathogenesis of COVID-19 associated ARDS and facilitate the discovery of novel interventions.

Future Studies:

- 1) **Impact of Sex, Age and Comorbidities:** Given the importance of sex considerations in disease development, future studies will compare differences in ALI response from VSV-CoV2-S administration between male and female K18-hACE2 transgenic mice. Moreover, it is widely recognized that COVID-19 severity and fatality rates are much greater in the elderly population and patients with comorbidities (e.g. obesity, cardiovascular disease, diabetes, etc.). Therefore, future studies will also evaluate the impact of mouse age and comorbidities on VSV-CoV2-S induced ALI.
- 2) **SARS-CoV2 Variants:** VSV-CoV2-S is a pseudotyped virus for the original SARS-CoV2. However, SARS-CoV2 variants including the Delta and Omega variants are now the dominant strains in North America. Given the increased infectivity and transmissibility, future studies will evaluate the virulence of established and newly rising variant strains using our VSV pseudovirus approach. This work will lead to a better understanding of how mutations within the spike protein may produce features of lung injury that vary from the original SARS-CoV2.
- 3) **Therapeutic Efficacy of MSCs and MSC-EVs:** Currently, Dr. Stewart and Dr. Lalu are leading the phase 2 Cellular Immuno-Therapy for COVID-19 ARDS Randomized Clinical Trial (CIRCA-19). In parallel, the efficacy of MSCs and MSC-EVs can be tested preclinically by utilizing our VSV-CoV2-S induced mouse model of COVID-19

ALI. If proven beneficial, these findings can provide more evidence for the therapeutic use of MSCs or MSC-EVs in treating patients with COVID-19 associated ARDS.

Appendix A – Supplemental Material for Chapter 2

Supplemental File A1 – PRISMA Checklist

This checklist has been adapted for use with systematic review protocol submissions to BioMed Central journals from Table 3 in Moher D et al: Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. Systematic Reviews 2015 4:1

Section/topic	#	Checklist item	Information reported		Line number(s)			
			Yes	No				
ADMINISTRATIVE INFORMATION								
Title								
Identification	1a	Identify the report as a protocol of a systematic review	☒	☐	Page 5, line 3			
Update	1b	If the protocol is for an update of a previous systematic review, identify as such	☒	☐	Page 5 line 9; Page 8 line 1			
Registration	2	If registered, provide the name of the registry (e.g., PROSPERO) and registration number in the Abstract	☒	☐	PROSPERO Registration Number Pending			
Authors								
Contact	3a	Provide name, institutional affiliation, and e-mail address of all protocol authors; provide physical mailing address of corresponding author	☒	☐	Page 1			
Contributions	3b	Describe contributions of protocol authors and identify the guarantor of the review	☒	☐	Page 12, line 13			
Amendments	4	If the protocol represents an amendment of a previously completed or published protocol, identify as such and list changes; otherwise, state plan for documenting important protocol amendments	☒	☐	Page 11, line 6			
Support								
Sources	5a	Indicate sources of financial or other support for the review	☒	☐	Page 12, line 19			

Section/topic	#	Checklist item	Information reported		Line number(s)
			Yes	No	
Sponsor	5b	Provide name for the review funder and/or sponsor	☒	☐	Page 12, line 19
Role of sponsor/funder	5c	Describe roles of funder(s), sponsor(s), and/or institution(s), if any, in developing the protocol	☒	☐	Page 12, line 19
INTRODUCTION					
Rationale	6	Describe the rationale for the review in the context of what is already known	☒	☐	Pages 4-5
Objectives	7	Provide an explicit statement of the question(s) the review will address with reference to participants, interventions, comparators, and outcomes (PICO)	☒	☐	Page 5, line 17
METHODS					
Eligibility criteria	8	Specify the study characteristics (e.g., PICO, study design, setting, time frame) and report characteristics (e.g., years considered, language, publication status) to be used as criteria for eligibility for the review	☒	☐	Page 6, line 16
Information sources	9	Describe all intended information sources (e.g., electronic databases, contact with study authors, trial registers, or other grey literature sources) with planned dates of coverage	☒	☐	Page 7, line 14
Search strategy	10	Present draft of search strategy to be used for at least one electronic database, including planned limits, such that it could be repeated	☒	☐	Page 7, line 19; Additional file 2
STUDY RECORDS					
Data management	11a	Describe the mechanism(s) that will be used to manage records and data throughout the review	☒	☐	Page 8, line 5
Selection process	11b	State the process that will be used for selecting studies (e.g., two independent reviewers) through each phase of the review (i.e., screening, eligibility, and inclusion in meta-analysis)	☒	☐	Page 8, line 9

Section/topic	#	Checklist item	Information reported		Line number(s)
			Yes	No	
Data collection process	11c	Describe planned method of extracting data from reports (e.g., piloting forms, done independently, in duplicate), any processes for obtaining and confirming data from investigators	☒	☐	Page 8, line 21
Data items	12	List and define all variables for which data will be sought (e.g., PICO items, funding sources), any pre-planned data assumptions and simplifications	☒	☐	Page 9, line 1
Outcomes and prioritization	13	List and define all outcomes for which data will be sought, including prioritization of main and additional outcomes, with rationale	☒	☐	Page 7, line 5
Risk of bias in individual studies	14	Describe anticipated methods for assessing risk of bias of individual studies, including whether this will be done at the outcome or study level, or both; state how this information will be used in data synthesis	☒	☐	Page 9, line 6
DATA					
Synthesis	15a	Describe criteria under which study data will be quantitatively synthesized	☒	☐	Page 9-10
	15b	If data are appropriate for quantitative synthesis, describe planned summary measures, methods of handling data, and methods of combining data from studies, including any planned exploration of consistency (e.g., I^2 , Kendall's tau)	☒	☐	Page 9, line 18;
	15c	Describe any proposed additional analyses (e.g., sensitivity or subgroup analyses, meta-regression)	☒	☐	Page 10, line 7
	15d	If quantitative synthesis is not appropriate, describe the type of summary planned	☒	☐	Page 10, line 24 to page 11, line 2
Meta-bias(es)	16	Specify any planned assessment of meta-bias(es) (e.g., publication bias across studies, selective reporting within studies)	☒	☐	Page 9, line 6
Confidence in cumulative evidence	17	Describe how the strength of the body of evidence will be assessed (e.g., GRADE)	☐	☒	Not applicable (GRADE does not exist for animal studies)

Supplemental File A2 – Search Strategy

Database: Ovid MEDLINE(R) ALL

Search Strategy:

-
- 1 Mesenchymal Stromal Cells/
 - 2 Mesenchymal Stem Cell Transplantation/
 - 3 Multipotent Stem Cells/
 - 4 **Acronyms:** (MSC or MSCs or ADMSC or ADMSCs or BM-MSC or BM-MSCs or BMD-MSC or BMD-MSCs or BMDMSC or BMDMSCs).tw,kw.
 - 5 (mesenchymal adj5 (cell* or stem or stromal or progenitor* or multipotent or bone marrow or adipose or placenta*)).tw.
 - 6 (mesenchymal and (cell* or stem or stromal or progenitor or multipotent or bone marrow or adipose or placenta*)).kw.
 - 7 ((multipotent or multi-potent) adj (stroma* cell* or stem cell*)).tw.
 - 8 ((multipotent or multi-potent) and (stroma* cell* or stem cell*)).kw.
 - 9 (colony-forming adj2 fibroblast*).tw.
 - 10 marrow stroma* cell*.tw.
 - 11 Mesoderm/cy
 - 12 or/1-11
 - 13 Exosomes/ or Extracellular Vesicles/
 - 14 exosom*.tw,kw.
 - 15 Cell-Derived Microparticles/
 - 16 (microvesicle* or micro vesicle*).tw,kw.
 - 17 ((cell or cells or extracellular) adj2 vesicle*).tw.
 - 18 microparticle*.tw,kw.
 - 19 ((cell or cells or extracellular) and vesicle*).kw.
 - 20 or/13-19
 - 21 12 and 20
 - 22 **Preclinical Filter:** exp "animal experimentation"/ or exp "models, animal"/ or exp "invertebrates"/ or "Animals"/ or exp "animal population groups"/ or "chordata"/ or exp "chordata, nonvertebrate"/ or "vertebrates"/ or exp "amphibians"/ or exp "birds"/ or exp "fishes"/ or exp "reptiles"/ or "mammals"/ or "primates"/ or exp "artiodactyla"/ or exp "carnivora"/ or exp "cetacea"/ or exp "chiroptera"/ or exp "elephants"/ or exp "hyraxes"/ or exp "insectivora"/ or exp "lagomorpha"/ or exp "marsupialia"/ or exp "monotremata"/ or exp "perissodactyla"/ or exp "rodentia"/ or exp "scandentia"/ or exp "sirenia"/ or exp "xenarthra"/ or "haplorhini"/ or exp "strepsirhini"/ or exp "platyrrhini"/ or exp "tarsii"/ or "catarrhini"/ or exp "cercopithecidae"/ or exp "hylobatidae"/ or "hominidae"/ or exp "gorilla gorilla"/ or exp "pan paniscus"/ or exp "pan troglodytes"/ or exp "pongo pygmaeus"/ (6252795)
 - 23 (animals or animal or mice or mus or mouse or murine or woodmouse or rats or rat or murinae or muridae or cottonrat or cottonrats or hamster or hamsters or cricetinae or rodentia or rodent or rodents or pigs or pig or swine or swines or piglets or piglet or boar or boars or "sus scrofa" or ferrets or ferret or polecat or polecats or "mustela putorius" or "guinea pigs" or "guinea pig" or cavia or callithrix or marmoset or marmosets or cebuella or hapale or octodon or chinchilla or chinchillas or gerbillinae or gerbil or gerbils or jird or jirds or merione or meriones or rabbits or rabbit or hares or hare or diptera or flies or fly or dipteral or drosophila or drosophilidae or cats or

cat or carus or felis or nematoda or nematode or nematoda or nematode or nematodes or sipunculida or dogs or dog or canine or canines or canis or sheep or sheeps or mouflon or mouflons or ovis or goats or goat or capra or capras or rupicapra or chamois or haplorhini or monkey or monkeys or anthropoidea or anthropoids or saguinus or tamarin or tamarins or leontopithecus or hominidae or ape or apes or pan or paniscus or "pan paniscus" or bonobo or bonobos or troglodytes or "pan troglodytes" or gibbon or gibbons or siamang or siamangs or nomascus or symphalangus or chimpanzee or chimpanzees or prosimians or "bush baby" or prosimian or bush babies or galagos or galago or pongidae or gorilla or gorillas or pongo or pygmaeus or "pongo pygmaeus" or orangutans or pygmaeus or lemur or lemurs or lemuridae or horse or horses or pongo or equus or cow or calf or bull or chicken or chickens or gallus or quail or bird or birds or quails or poultry or poultries or fowl or fowls or reptile or reptilia or reptiles or snakes or snake or lizard or lizards or alligator or alligators or crocodile or crocodiles or turtle or turtles or amphibian or amphibians or amphibia or frog or frogs or bombina or salientia or toad or toads or "epidalea calamita" or salamander or salamanders or eel or eels or fish or fishes or pisces or catfish or catfishes or siluriformes or arius or heteropneustes or sheatfish or perch or perches or percidae or perca or trout or trouts or char or chars or salvelinus or "fathead minnow" or minnow or cyprinidae or carps or carp or zebrafish or zebrafishes or goldfish or goldfishes or guppy or guppies or chub or chubs or tinca or barbels or barbus or pimephales or promelas or "poecilia reticulata" or mullet or mullets or seahorse or seahorses or mugil curema or atlantic cod or shark or sharks or catshark or anguilla or salmonid or salmonids or whitefish or whitefishes or salmon or salmons or sole or solea or "sea lamprey" or lamprey or lampreys or pumpkinseed or sunfish or sunfishes or tilapia or tilapias or turbot or turbots or flatfish or flatfishes or sciuridae or squirrel or squirrels or chipmunk or chipmunks or suslik or susliks or vole or voles or lemming or lemmings or muskrat or muskrats or lemmus or otter or otters or marten or martens or martes or weasel or badger or badgers or ermine or mink or minks or sable or sables or gulo or gulos or wolverine or wolverines or minks or mustela or llama or llamas or alpaca or alpacas or camelid or camelids or guanaco or guanacos or chiroptera or chiropteras or bat or bats or fox or foxes or iguana or iguanas or xenopus laevis or parakeet or parakeets or parrot or parrots or donkey or donkeys or mule or mules or zebra or zebras or shrew or shrews or bison or bisons or buffalo or buffaloes or deer or deers or bear or bears or panda or pandas or "wild hog" or "wild boar" or fitchew or fitch or beaver or beavers or jerboa or jerboas or capybara or capybaras).tw.

- 24 Drug Evaluation, Preclinical/
- 25 (preclinic* or pre clinic*).tw,kw.
- 26 22 or 23 or 24 or 25
- 27 21 and 26

Database: Embase Classic+Embase
Search Strategy:

-
- 1 exp mesenchymal stem cell/
 - 2 exp mesenchymal stem cell transplantation/

- 3 exp mesenchymal stroma cell/
- 4 **Acronyms:** (MSC or MSCs or ADMSC or ADMSCs or BM-MSC or BM-MSCs or BMD-MSC or BMD-MSCs or BMDMSC or BMDMSCs or BMSC or BMSCs or HBMSC or HBMSCs or ABMSC or ABMSCs or MAPC or MAPCs).tw.
- 5 (((multipotent or multi-potent) adj3 (stem or stroma\$1 or progenitor*)) and (cell\$1 or cell-induced)).tw.
- 6 multipotent stem cell/
- 7 (mesenchymal adj5 (cell* or stem or stroma* or progenitor* or multipotent or bone marrow or adipose or placenta*)).tw.
- 8 (colony-forming adj2 fibroblast*).tw.
- 9 (marrow stroma* adj2 cell*).tw.
- 10 or/1-9
- 11 exosome/
- 12 exosom*.tw.
- 13 (microvesicle* or micro vesicle*).tw.
- 14 microparticle*.tw.
- 15 ((cell or cells or extracellular) adj2 vesicle*).tw.
- 16 exp membrane microparticle/
- 17 or/11-16
- 18 10 and 17
- 19 exp animal experiment/ or exp animal model/ or exp experimental animal/ or exp transgenic animal/ or exp male animal/ or exp female animal/ or exp juvenile animal/ or animal/ or chordata/ or vertebrate/
- 20 tetrapod/ or exp fish/ or amniote/ or exp amphibia/ or mammal/ or exp reptile/ or exp sauropsid/ or therian/ or exp monotremate/ or placental mammals/ or exp marsupial/ or Euarchontoglires/ or exp Afrotheria/ or exp Boreoeutheria/ or exp Laurasiatheria/ or exp Xenarthra/ or primate/ or exp Dermoptera/ or exp Glires/ or exp Scandentia/ or Haplorhini/ or exp prosimian/ or simian/ or exp tarsiiiform/ or Catarrhini/ or exp Platyrrhini/ or ape/ or exp Cercopithecidae/ or hominid/ or exp hylobatidae/ or exp chimpanzee/ or exp gorilla/ or exp orang utan/
- 21 (preclinic* or pre clinic*).tw.
- 22 **Preclinical Filter:** (animal or animals or pisces or fish or fishes or catfish or catfishes or sheatfish or silurus or arius or heteropneustes or clarias or gariepinus or fathead minnow or fathead minnows or pimephales or promelas or cichlidae or trout or trouts or char or chars or salvelinus or salmo or oncorhynchus or guppy or guppies or millionfish or poecilia or goldfish or goldfishes or carassius or auratus or mullet or mullets or mugil or curema or shark or sharks or cod or cods or gadus or morhua or carp or carps or cyprinus or carpio or killifish or eel or eels or anguilla or zander or sander or lucioperca or stizostedion or turbot or turbots or psetta or flatfish or flatfishes or plaice or pleuronectes or platessa or tilapia or tilapias).tw.
- 23 **Preclinical Filter:** (oreochromis or sarotherodon or common sole or dover sole or solea or zebrafish or zebrafishes or danio or rerio or seabass or dicentrarchus or labrax or morone or lamprey or lampreys or petromyzon or pumpkinseed or pumpkinseeds or lepomis or gibbosus or herring or clupea or harengus or amphibia or amphibian or amphibians or anura or salientia or frog or frogs or rana or toad or toads or bufo or xenopus or laevis or bombina or epidalea or calamita or salamander or salamanders or newt or newts or triturus or reptilia or reptile or reptiles or bearded dragon or pogona or vitticeps or iguana or iguanas or lizard).tw.

24 **Preclinical Filter:** (lizards or anguis fragilis or turtle or turtles or snakes or snake or aves or bird or birds or quail or quails or coturnix or bobwhite or colinus or virginianus or poultry or poultries or fowl or fowls or chicken or chickens or gallus or zebra finch or taeniopygia or guttata or canary or canaries or serinus or canaria or parakeet or parakeets or grasskeet or parrot or parrots or psittacine or psittacines or shelduck or tadorna or goose or geese or branta or leucopsis or woodlark or lullula or flycatcher or ficedula or hypoleuca or dove or doves or geopelia or cuneata or duck or ducks or greylag or graylag or anser or harrier or circus pygargus or red knot or great knot or calidris or canutus or godwit or limosa or lapponica or meleagris or gallopavo or jackdaw or corvus or monedula or ruff or philomachus or pugnax or lapwing or peewit or plover or vanellus or swan or cygnus or columbianus or bewickii or gull or chroicocephalus or ridibundus).tw.

25 **Preclinical Filter:** (albifrons or great tit or parus or aythya or fuligula or streptopelia or risoria or spoonbill or platalea or leucorodia or blackbird or turdus or merula or blue tit or cyanistes or pigeon or pigeons or columba or pintail or anas or starling or sturnus or owl or athene noctua or pochard or ferina or cockatiel or nymphicus or hollandicus or skylark or alauda or tern or sterna or teal or crecca or oystercatcher or haematopus or ostralegus or shrew or shrews or sorex or araneus or crocidura or russula or european mole or talpa or chiroptera or bat or bats or eptesicus or serotinus or myotis or dasycneme or daubentonii or pipistrelle or pipistrellus or cat or cats or felis or catus or feline or dog or dogs or canis or canine or canines or otter or otters or lutra or badger or badgers or meles or fitchew or fitch or foumart or foulmart or ferrets or ferret or polecat or polecats or mustela or putorius or weasel or weasels or fox or foxes or vulpes or common seal or phoca or vitulina or grey seal or halichoerus or horse or horses or equus or equine or equidae or donkey or donkeys or mule or mules or pig or pigs or swine or swines or hog or hogs or boar or boars or porcine or piglet or piglets or sus or scrofa or llama or llamas or lama or glama or deer or deers or cervus or elaphus or cow or cows or bos taurus or bos indicus or bovine or bull or bulls).tw.

26 (cattle or bison or bisons or sheep or sheeps or ovis aries or ovine or lamb or lambs or mouflon or mouflons or goat or goats or capra or caprine or chamois or rupicapra or leporidae or lagomorpha or lagomorph or rabbit or rabbits or oryctolagus or cuniculus or laprine or hares or lepus or rodentia or rodent or rodents or murinae or mouse or mice or mus or musculus or murine or woodmouse or apodemus or rat or rats or rattus or norvegicus or guinea pig or guinea pigs or cavia or porcellus or hamster or hamsters or mesocricetus or cricetus or cricetus or gerbil or gerbils or jird or jirds or meriones or unguiculatus or jerboa or jerboas or jaculus or chinchilla or chinchillas or beaver or beavers or castor fiber or castor canadensis or sciuridae or squirrel or squirrels or sciurus or chipmunk or chipmunks or marmot or marmots or marmota or suslik or susliks or spermophilus or cynomys or cottonrat or cottonrats or sigmodon or vole or voles or microtus or myodes or glareolus or primate).tw.

27 **Preclinical Filter:** (primates or prosimian or prosimians or lemur or lemurs or lemuridae or loris or bush baby or bush babies or bushbaby or bushbabies or galago or galagos or anthropoidea or anthropoids or simian or simians or monkey or monkeys or marmoset or marmosets or callithrix or cebuella or tamarin or tamarins or saguinus or leontopithecus or squirrel monkey or squirrel monkeys or saimiri or night monkey or night monkeys or owl monkey or owl monkeys or douroucoulis or aotus or spider monkey or spider monkeys or ateles or baboon or baboons or papio or rhesus monkey or macaque or macaca or mulatta or cynomolgus or fascicularis or green monkey or green monkeys or chlorocebus or vervet or vervets or pygerythrus or hominoidea or ape or apes or hylobatidae or gibbon or gibbons or siamang or siamangs or nomascus or symphalangus or hominidae or orangutan or orangutans or pongo or chimpanzee or chimpanzees or pan troglodytes or bonobo or bonobos or pan paniscus or gorilla or gorillas or troglodytes).tw.

28 or/19-27
29 18 and 28
30 conference abstract.pt.
31 29 not 30

Appendix B – Supplemental Material for Chapter 3

Supplemental File B1 – Reference List for 206 Included Studies

1. Ahn, S. Y.; Park, W. S.; Kim, Y. E.; Sung, D. K.; Sung, S. I.; Ahn, J. Y.; Chang, Y. S., Vascular Endothelial Growth Factor Mediates the Therapeutic Efficacy of Mesenchymal Stem Cell-Derived Extracellular Vesicles against Neonatal Hyperoxic Lung Injury. *Exp. Mol. Med.* **2018**, *50*, 1-12.
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Supplemental File B2 – Interventional Details

<u>Publication Year</u>	<u>First Author's Last Name</u>	<u>Country of Corresponding Author</u>	<u>Terminology Used</u>	<u>Size of EVs</u>	<u>Isolation Technique</u>	<u>Species Source of MSCs</u>	<u>Tissue Source of MSCs</u>	<u>EV Immunocompatibility</u>	<u>Were EVs Modified?</u>
2009	Bruno	Italy	Microvesicles	Both used Together	Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Modified
2010	Lai	Singapore	Microparticles	Small EVs (30-150nm)	Size Exclusion Chromatography	Human Source	Fetal	Xenogeneic	Unmodified
2011	Chen	Singapore	Exosomes	Small EVs (30-150nm)	Size Exclusion Chromatography	Human Source	Embryonic Stem Cell-derived MSCs	Xenogeneic	Modified
2011	Gatti	Italy	Microvesicles	Both used Together	Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Modified
2012	Lai	Singapore	Exosomes	Small EVs (30-150nm)	Tangential Flow Filtration, Size Exclusion Chromatography	Human Source	Embryonic Stem Cell-derived MSCs	Xenogeneic	Unmodified
2012	Lee	United States	Exosomes	Small EVs (30-150nm)	Isolation Kit, Ultracentrifugation, Ultrafiltration, Size Exclusion Chromatography	Mouse Source	Bone Marrow	Allogeneic	Unmodified
2012	Reis	Brazil	Exosomes, Microvesicles	Not Described	Ultracentrifugation	Rat Source	Bone Marrow	Allogeneic	Modified
2012	Zhang	China	Microvesicles	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified
2012	van Koppen	Netherlands	Exosomes	Not Described	Ultracentrifugation, Sucrose Gradient Purification, Ultrafiltration	Human Source	Embryonic Stem Cell-derived MSCs	Xenogeneic	Unmodified
2012	Bruno	Italy	Microvesicles	Both used Together	Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Modified
2012	He	China	Microvesicles	Small EVs (30-150nm)	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Unmodified
2013	Kilpinen	Finland	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Human Source	Cord Blood	Xenogeneic	Modified
2013	Lee	South Korea	Exosomes	Not Described	Isolation Kit	Mouse Source	Bone Marrow	Allogeneic	Unmodified
2013	Xin	United States	Exosomes	Not Described	Ultracentrifugation	Rat Source	Bone Marrow	Allogeneic	Unmodified
2013	Zhou	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified

2013	Wu	China	Microvesicles	Both used Together	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2013	Katakowski	United States	Exosomes	Not Described	Isolation Kit	Not described	Bone Marrow	Not described	Modified
2013	Arslan	Netherlands	Exosomes	Not Described	Tangential Flow Filtration, Size Exclusion Chromatography	Human Source	Embryonic Stem Cell-derived MSCs	Xenogeneic	Unmodified
2013	Li	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2013	Roccaro	United States	Exosomes	Small EVs (30-150nm)	Isolation Kit, Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Modified
2013	Bruno	Italy	Microvesicles	Small EVs (30-150nm)	Ultracentrifugation, Ultrafiltration	Human Source	Bone Marrow	Xenogeneic	Unmodified
2014	Chen	United States	Microvesicles	Both used Together	Ultracentrifugation	Rat Source	Bone Marrow	Allogeneic	Unmodified
2014	Feng	China	Exosomes	Small EVs (30-150nm)	Isolation Kit	Human Source	Bone Marrow	Xenogeneic	Unmodified
2014	Tan	Singapore	Exosomes	Not Described	Tangential Flow Filtration, Size Exclusion Chromatography	Human Source	Embryonic Stem Cell-derived MSCs	Xenogeneic	Unmodified
2014	Du	China	Microvesicles	Both used Together	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified
2014	Raisi	Iran	Microvesicles	Not Described	Ultracentrifugation	Rat Source	Adipose	Allogeneic	Modified
2014	Zou	China	Microvesicles	Both used Together	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2014	Zhang	China	Microvesicles	Both used Together	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2014	Choi	South Korea	Microvesicles	Not Described	Isolation Kit	Human Source	Bone Marrow	Xenogeneic	Unmodified
2014	Feng	United States	Exosomes	Small EVs (30-150nm)	Isolation Kit, Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Modified
2014	Choi	South Korea	Microparticles	Not Described	Ultracentrifugation	Mouse Source	Kidney	Allogeneic	Modified
2014	Zhang	Singapore	Exosomes	Not Described	Tangential Flow Filtration, Size Exclusion Chromatography	Human Source	Embryonic Stem Cell-derived MSCs	Xenogeneic	Unmodified
2014	Bian	China	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Modified

2014	Rahman	United States	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Mouse Source	pancreatic islet MSC-like cells	Allogeneic	Unmodified
2014	Zhu	United States	Microvesicles	Large EVs (150-1000nm)	Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Modified
2015	Koch	Germany	Extracellular Vesicles (EVs)	Not Described	Ultracentrifugation	Rat Source	Bone Marrow	Allogeneic	Unmodified
2015	Zhao	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2015	Kang	United States	Exosomes	Small EVs (30-150nm)	Isolation Kit	Rat Source	Bone Marrow	Allogeneic	Modified
2015	Li	China	Exosomes	Not Described	Isolation Kit	Human Source	Bone Marrow	Xenogeneic	Modified
2015	Yang	China	Exosomes	Small EVs (30-150nm)	Isolation Kit	Rat Source	Not Described	Allogeneic	Modified
2015	Teng	China	Exosomes	Small EVs (30-150nm)	Isolation Kit	Rat Source	Bone Marrow	Allogeneic	Unmodified
2015	Lou	China	Exosomes	Not Described	Isolation Kit	Human Source	Adipose	Xenogeneic	Modified
2015	Yang	China	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Rat Source	Bone Marrow	Allogeneic	Unmodified
2015	Geiger	Germany	Exosomes	Small EVs (30-150nm)	Ultrafiltration	Human Source	Fibrocytes	Xenogeneic	Unmodified
2015	Ti	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified
2015	Cruz	United States	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Human Source, Mouse Source	Bone Marrow	Allogeneic, Xenogeneic	Unmodified
2015	Wang	United States	Exosomes	Small EVs (30-150nm)	Ultracentrifugation, Sucrose Gradient Purification	Mouse Source	Bone Marrow	Allogeneic	Modified
2015	Doeppner	Germany	Extracellular Vesicles (EVs)	Not Described	Isolation Kit, Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Unmodified
2015	Hu	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation, Ultrafiltration	Human Source	iPSC-derived MSCs	Xenogeneic	Unmodified
2015	Monsel	United States	Microvesicles	Large EVs (150-1000nm)	Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Modified
2015	Wang	China	Microvesicles	Not Described	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Modified
2015	Choi	South Korea	Microparticles	Not Described	Ultracentrifugation	Mouse Source	Kidney	Allogeneic	Modified
2015	Nakamura	Japan	Exosomes	Small EVs (30-150nm)	Ultracentrifugation, Ultrafiltration	Human Source	Bone Marrow	Xenogeneic	Unmodified
2015	Zhang	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation, Hollow Fiber	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified

				Membrane, Sucrose Cushion					
2015	Ju	China	Microvesicles	Both used Together	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified
2015	Zhang	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	iPSC-derived MSCs	Xenogeneic	Unmodified
2015	Yu	United States	Exosomes	Small EVs (30-150nm)	Isolation Kit, Ultrafiltration	Rat Source	Bone Marrow	Allogeneic	Modified
2015	Zhang	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified
2015	Ko	Taiwan	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Rat Source	Adipose	Allogeneic	Unmodified
2015	Zhang	United States	Exosomes	Small EVs (30-150nm)	Isolation Kit	Rat Source	Bone Marrow	Allogeneic	Unmodified
2015	Collino	Italy	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Modified
2016	Yu	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source, Mouse Source	Adipose, Umbilical Cord/Wharton's Jelly	Allogeneic, Xenogeneic	Modified
2016	Tamura	Japan	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Unmodified
2016	Shen	China	Exosomes	Not Described	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Modified
2016	Zou	China	Extracellular Vesicles (EVs)	Large EVs (150-1000nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified
2016	Gu	China	Extracellular Vesicles (EVs)	Not Described	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified
2016	Nagaishi	Japan	Exosomes	Small EVs (30-150nm)	Isolation Kit	Rat Source	Bone Marrow	Allogeneic	Unmodified
2016	Hu	China	Exosomes	Small EVs (30-150nm)	Isolation Kit	Human Source	Adipose	Xenogeneic	Unmodified
2016	Lee	South Korea	Microvesicles	Not Described	Tangential Flow Filtration, Ultracentrifugation	Human Source	Adipose	Xenogeneic	Modified
2016	Xie	China	Microvesicles	Both used Together	Ultracentrifugation	Rat Source	Bone Marrow	Xenogeneic	Modified
2016	Zhang	Singapore	Exosomes	Small EVs (30-150nm)	Size Exclusion Chromatography	Human Source	Embryonic Stem Cell-derived MSCs	Xenogeneic	Unmodified
2016	Yin	China	Microvesicles	Not Described	Ultracentrifugation	Rat Source	Bone Marrow	Allogeneic, Xenogeneic	Unmodified
2016	Kalimuthu	South Korea	Extracellular Vesicles (EVs)	Small EVs (30-150nm)	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Modified

2016	Chen	Taiwan	Exosomes	Not Described	Ultracentrifugation	Pig Source	Adipose	Xenogeneic	Modified
2016	Nakano	Japan	Exosomes	Small EVs (30-150nm)	Ultracentrifugation, Sucrose Gradient Purification	Rat Source	Bone Marrow	Xenogeneic	Unmodified
2016	Wen	United States	Exosomes	Not Described	Isolation Kit	Human Source	Bone Marrow	Xenogeneic	Modified
2016	Liu	United States	Microvesicles	Large EVs (150-1000nm)	Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Modified
2016	Zhang	China	Exosomes	Not Described	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified
2016	Zou	China	Extracellular Vesicles (EVs)	Large EVs (150-1000nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2016	Wen	United States	Extracellular Vesicles (EVs)	Large EVs (150-1000nm), Both used Together	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Unmodified
2016	Guo	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation, Ultrafiltration	Human Source	Synovial membrane	Xenogeneic	Unmodified
2016	Nong	China	Exosomes	Small EVs (30-150nm)	Ultrafiltration	Human Source	iPSC-derived MSCs	Xenogeneic	Unmodified
2016	Furuta	Japan	Exosomes	Small EVs (30-150nm)	Ultracentrifugation, Ultrafiltration	Human Source	Bone Marrow	Xenogeneic	Unmodified
2016	Hu	China	Extracellular Vesicles (EVs)	Not Described	Isolation Kit	Human Source	Bone Marrow	Xenogeneic	Unmodified
2016	Vrijssen	Netherlands	Exosomes	Small EVs (30-150nm)	Ultracentrifugation, Sucrose Gradient Purification	Human Source	Bone Marrow	Xenogeneic	Modified
2016	Li	China	Exosomes	Small EVs (30-150nm)	Isolation Kit	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified
2016	Fang	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified
2016	Qi	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation, Ultrafiltration	Human Source	iPSC-derived MSCs	Xenogeneic	Modified
2016	Ophelders	Netherlands	Extracellular Vesicles (EVs)	Not Described	Isolation Kit	Human Source	Bone Marrow	Xenogeneic	Unmodified
2016	Lin	Taiwan	Exosomes	Not Described	Ultracentrifugation	Rat Source	Adipose	Allogeneic	Unmodified
2016	Rager	United States	Exosomes	Not Described	Isolation Kit	Mouse Source	Bone Marrow	Xenogeneic	Unmodified
2016	Aliotta	United States	Exosomes, Microvesicles, Extracellular Vesicles (EVs)	Small EVs (30-150nm), Both used Together	Ultracentrifugation	Human Source, Mouse Source	Bone Marrow	Allogeneic, Xenogeneic	Unmodified

2016	Qin	China	Extracellular Vesicles (EVs)	Not Described	Ultracentrifugation, Ultrafiltration	Human Source	Bone Marrow	Xenogeneic	Modified
2016	Zhang	China	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2016	Sun	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2016	Kim	United States	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation, Size Exclusion Chromatography	Human Source	Bone Marrow	Xenogeneic	Unmodified
2016	Liu	China	Extracellular Vesicles (EVs)	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Adipose	Xenogeneic	Unmodified
2016	Wang	China	Extracellular Vesicles (EVs)	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2016	Ti	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified
2016	Shi	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Unmodified
2016	Lange	Germany	Microvesicles	Both used Together	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Unmodified
2017	Fujii	Japan	Extracellular Vesicles (EVs)	Both used Together	Isolation Kit	Human Source	Bone Marrow	Xenogeneic	Unmodified
2017	Cheng	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Modified
2017	Willis	United States	Exosomes	Small EVs (30-150nm)	Tangential Flow Filtration, Flotation on Iodixanol	Human Source	Bone Marrow, Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2017	Zhu	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Modified
2017	Webb	United States	Extracellular Vesicles (EVs)	Both used Together	Ultrafiltration	Human Source	Embryonic Stem Cell-derived MSCs	Xenogeneic	Unmodified
2017	Wang	China	Exosomes	Not Described	Isolation Kit	Human Source	Adipose	Xenogeneic	Unmodified
2017	Mead	United States	Exosomes	Not Described	Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Modified
2017	Yuan	China	Extracellular Vesicles (EVs)	Small EVs (30-150nm)	Isolation Kit	Human Source	iPSC-derived MSCs	Xenogeneic	Modified
2017	Soundara Rajan	Italy	Exosomes, Microvesicles	Not Described	Isolation Kit	Human Source	Periodontal ligament	Xenogeneic	Unmodified
2017	El-Tookhy	Egypt	Exosomes, Microvesicles	Not Described	Ultracentrifugation	Dog Source	Bone Marrow	Autologous	Unmodified
2017	Schoefinius	Germany	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Unmodified

2017	Huang	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Rat Source	Bone Marrow	Allogeneic	Unmodified
2017	Chen	China	Microvesicles	Not Described	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified
2017	Zhang	United States	Exosomes	Not Described	Isolation Kit	Human Source	Bone Marrow	Xenogeneic	Modified
2017	Stone	United States	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2017	Horn	Germany	Extracellular Vesicles (EVs)	Not Described	Isolation Kit, Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Unmodified
2017	Cosenza	France	Exosomes, Microparticles	Both used Separately	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Unmodified
2017	Shi	China	Exosomes	Small EVs (30-150nm)	Size Exclusion Chromatography	Human Source	Gingival tissue	Xenogeneic	Modified
2017	Rajendran	South Korea	Extracellular Vesicles (EVs)	Small EVs (30-150nm)	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Unmodified
2017	Bruno	Italy	Extracellular Vesicles (EVs)	Both used Together, Both used Separately	Ultracentrifugation, Flotation on Iodixanol	Human Source	Bone Marrow	Xenogeneic	Unmodified
2017	Aliotta	United States	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Unmodified
2017	Komaki	Japan	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Placenta	Xenogeneic	Unmodified
2017	Li	China	Exosomes	Small EVs (30-150nm)	Isolation Kit	Human Source	Exfoliated deciduous teeth	Xenogeneic	Unmodified
2017	Gangadaran	South Korea	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation, Flotation on Iodixanol	Mouse Source	Bone Marrow	Allogeneic	Modified
2017	Deng	China	Extracellular Vesicles (EVs)	Not Described	Isolation Kit	Mouse Source	Bone Marrow	Allogeneic	Unmodified
2017	Bai	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2017	Sun	Taiwan	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Pig Source	Adipose	Xenogeneic	Modified
2017	Gong	United States	Exosomes	Small EVs (30-150nm)	Isolation Kit	Mouse Source	Embryonic Stem Cell-derived MSCs	Allogeneic	Modified
2017	Qu	China	Exosomes	Not Described	Isolation Kit	Mouse Source	Adipose	Allogeneic	Modified
2017	Casado	Spain	Exosomes	Both used Together	Ultrafiltration	Pig Source	Bone Marrow	Not described	Modified
2017	Xie	China	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Rat Source	Bone Marrow	Xenogeneic	Modified

2017	Otero-Ortega	Spain	Extracellular Vesicles (EVs)	Small EVs (30-150nm)	Isolation Kit	Rat Source	Adipose	Allogeneic	Unmodified
2017	Wang	China	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Modified
2017	Haga	United States	Extracellular Vesicles (EVs)	Small EVs (30-150nm)	Ultracentrifugation	Human Source, Mouse Source	Bone Marrow	Allogeneic, Xenogeneic	Unmodified
2017	Ma	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified
2017	Wang	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Embryonic Stem Cell-derived MSCs	Xenogeneic	Unmodified
2017	Long	United States	Exosomes, Extracellular Vesicles (EVs)	Small EVs (30-150nm)	Size Exclusion Chromatography	Human Source	Bone Marrow	Xenogeneic	Unmodified
2017	Li	China	Exosomes	Small EVs (30-150nm)	Isolation Kit	Rabbit Source	Bone Marrow	Allogeneic	Modified
2017	Eirin	United States	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Pig Source	Adipose	Autologous	Modified
2017	de Castro	Brazil	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Human Source	Adipose	Xenogeneic	Unmodified
2017	Tang	China	Microvesicles	Large EVs (150-1000nm)	Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Modified
2017	Gonzalez-King	Spain	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Dental pulp	Xenogeneic	Modified
2017	Haga	United States	Extracellular Vesicles (EVs)	Small EVs (30-150nm)	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Unmodified
2017	Mao	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2017	Shigemoto-Kuroda	United States	Extracellular Vesicles (EVs)	Small EVs (30-150nm)	Size Exclusion Chromatography	Human Source	Bone Marrow	Xenogeneic	Unmodified
2017	Du	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Placenta	Xenogeneic	Modified
2017	Chen	China	Exosomes	Small EVs (30-150nm)	Isolation Kit	Human Source	Menstrual blood	Xenogeneic	Unmodified
2017	Song	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified
2017	Moisseiev	United States	Exosomes	Not Described	Tangential Flow Filtration	Human Source	Bone Marrow	Xenogeneic	Modified
2017	Liu	China	Exosomes	Small EVs (30-150nm)	Isolation Kit	Rat Source	Bone Marrow	Allogeneic	Modified
2017	Zhu	China	Exosomes	Small EVs (30-150nm)	Ultrafiltration	Human Source	iPSC-derived MSCs, Synovial membrane	Xenogeneic	Unmodified

2017	Xin	United States	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Rat Source	Bone Marrow	Allogeneic	Modified
2017	Yan	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation, Ultrafiltration	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified
2017	Tao	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation, Ultrafiltration	Human Source	Synovial membrane	Xenogeneic	Modified
2017	Liu	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	iPSC-derived MSCs	Xenogeneic	Unmodified
2017	Kamerkar	United States	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	human foreskin fibroblast	Xenogeneic	Modified
2017	Du	China	Exosomes	Small EVs (30-150nm)	Isolation Kit	Human Source	iPSC-derived MSCs	Xenogeneic	Modified
2017	Wang	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2017	Zhao	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Amniotic	Xenogeneic	Unmodified
2017	Shao	China	Exosomes	Not Described	Ultracentrifugation	Rat Source	Bone Marrow	Allogeneic	Unmodified
2017	Tao	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation, Ultrafiltration	Human Source	Synovial membrane	Xenogeneic	Modified
2017	Huang	China	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Rat Source	Bone Marrow	Xenogeneic	Unmodified
2017	Tamura	Japan	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Modified
2017	Grigorian-Shamagian	United States	Extracellular Vesicles (EVs)	Both used Together	Isolation Kit	Human Source	Bone Marrow	Xenogeneic	Unmodified
2017	Ranghino	Italy	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Human Source	Kidney	Xenogeneic	Modified
2017	Cui	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation, Ultrafiltration	Rat Source	Adipose	Allogeneic	Unmodified
2017	Henao Agudelo	Brazil	Microvesicles	Small EVs (30-150nm)	Ultracentrifugation	Mouse Source	Adipose	Allogeneic	Unmodified
2017	Xin	United States	Exosomes	Not Described	Ultracentrifugation	Rat Source	Bone Marrow	Allogeneic	Modified
2017	Lo Sicco	Italy	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Human Source	Adipose	Xenogeneic	Modified
2017	de Almeida	Brazil	Microvesicles	Small EVs (30-150nm)	Ultracentrifugation	Mouse Source	Adipose	Allogeneic	Modified
2018	Luther	United States	Exosomes	Not Described	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Modified
2018	Williams	United States	Exosomes	Not Described	Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Unmodified
2018	McCulloh	United States	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Rat Source	Bone Marrow, Amniotic	Allogeneic	Unmodified

2018	Zhang	Singapore	Exosomes	Not Described	Tangential Flow Filtration	Human Source	Embryonic Stem Cell-derived MSCs	Xenogeneic	Unmodified
2018	Barile	Switzerland	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Unmodified
2018	Otero-Ortega	Spain	Exosomes	Small EVs (30-150nm)	Isolation Kit	Rat Source	Adipose	Allogeneic	Unmodified
2018	Ahn	South Korea	Extracellular Vesicles (EVs)	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Cord Blood	Xenogeneic	Modified
2018	Jiang	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2018	Diomedea	Italy	Extracellular Vesicles (EVs)	Both used Together	Isolation Kit	Human Source	Gingival tissue	Xenogeneic	Modified
2018	He	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Cord Blood	Xenogeneic	Unmodified
2018	Blazquez	Spain	Exosomes	Small EVs (30-150nm)	Ultrafiltration	Mouse Source	Bone Marrow	Allogeneic	Modified
2018	Shen	China	Exosomes	Not Described	Isolation Kit	Rat Source	Bone Marrow	Allogeneic	Modified
2018	Lang	United States	Exosomes	Small EVs (30-150nm)	Ultracentrifugation, Sucrose Gradient Purification	Human Source	Bone Marrow	Xenogeneic	Modified
2018	Wang	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2018	Cui	China	Exosomes	Not Described	Isolation Kit	Mouse Source	Bone Marrow	Allogeneic	Modified
2018	Damania	India	Exosomes	Both used Together	Ultracentrifugation, Ultrafiltration	Rat Source	Bone Marrow	Allogeneic	Unmodified
2018	Li	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Adipose	Xenogeneic	Modified
2018	Zhu	China	Exosomes	Small EVs (30-150nm)	Isolation Kit	Rat Source	Adipose	Allogeneic	Unmodified
2018	Khatri	United States	Extracellular Vesicles (EVs)	Small EVs (30-150nm)	Ultracentrifugation	Pig Source	Bone Marrow	Allogeneic	Unmodified
2018	Mead	United States	Small EV	Small EVs (30-150nm)	Isolation Kit	Human Source	Bone Marrow	Xenogeneic	Modified
2018	Wang	China	Extracellular Vesicles (EVs)	Not Described	Isolation Kit	Mouse Source	Bone Marrow	Allogeneic	Unmodified
2018	Otsuru	United States	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Unmodified
2018	Wu	China	Microvesicles	Not Described	Ultracentrifugation	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Unmodified
2018	Potter	United States	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Unmodified

2018	Ruppert	United States	Extracellular Vesicles (EVs)	Not Described	Tangential Flow Filtration	Human Source	Bone Marrow	Xenogeneic	Modified
2018	Han	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Adipose	Xenogeneic	Modified
2018	Chen	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Modified
2018	Mardpour	Iran	Extracellular Vesicles (EVs)	Both used Together	Ultracentrifugation	Human Source	Embryonic Stem Cell-derived MSCs	Xenogeneic	Unmodified
2018	O'Brien	Ireland	Exosomes, Extracellular Vesicles (EVs)	Small EVs (30-150nm)	Ultracentrifugation	Human Source	Bone Marrow	Xenogeneic	Modified
2018	Chang	China	Exosomes	Not Described	Ultracentrifugation	Rat Source	Adipose	Allogeneic	Modified
2018	Tian	China	Exosomes	Small EVs (30-150nm)	Ultracentrifugation	Rabbit Source	Bone Marrow	Not described	Modified
2018	Kou	United States	Exosomes, Small EV	Not Described	Ultracentrifugation, Sucrose Cushion	Mouse Source	Gingival tissue, Skin MSCs	Allogeneic	Modified
2018	Chen	China	Exosomes	Small EVs (30-150nm)	Isolation Kit, Ultrafiltration	Human Source	Urine	Xenogeneic	Modified
2018	Jia	China	Exosomes	Small EVs (30-150nm)	Isolation Kit	Human Source	Umbilical Cord/Wharton's Jelly	Xenogeneic	Modified
2018	Cosenza	France	Exosomes, Extracellular Vesicles (EVs), Microparticles	Both used Together, Both used Separately	Ultracentrifugation	Mouse Source	Bone Marrow	Allogeneic	Unmodified

Supplemental File B3 – Outcomes and Animal Models

<u>Year</u>	<u>First Author's Last Name</u>	<u>Disease Domain</u>	<u>Species of Animal Model</u>	<u>Treatment or Prevention Protocol</u>	<u>Were In Vivo Biodistribution Studies Performed?</u>	<u>Was Randomization Conducted?</u>	<u>Are Benefits Reported?</u>
2009	Bruno	Renal	Mouse	Post-injury induction (Treatment)	Yes	Not Described	Yes
2010	Lai	Cardiac	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2011	Chen	Cardiac	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2011	Gatti	Renal	Rat	Post-injury induction (Treatment)	Yes	Not Described	Yes
2012	Lai	Cardiac	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2012	Lee	Lung	Mouse	Both Prevention and Treatment Protocols	No	Not Described	Yes
2012	Reis	Renal	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2012	Zhang	Ischemia (e.g. testing angiogenesis)	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2012	van Koppen	Renal	Rat	Post-injury induction (Treatment)	No	Not Described	No benefits
2012	Bruno	Renal	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2012	He	Renal	Mouse	Post-injury induction (Treatment)	No	Yes	Mixed
2013	Kilpinen	Renal	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2013	Lee	Cancer	Mouse	At the same time as injury induction	No	Yes	Yes
2013	Xin	Neurological	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2013	Zhou	Renal	Rat	Post-injury induction (Treatment)	Yes	Not Described	Yes
2013	Wu	Cancer	Mouse	At the same time as injury induction	No	Yes	Yes
2013	Katakowski	Cancer	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2013	Arslan	Cardiac	Mouse	Pre-injury induction (Prevention)	No	Not Described	Mixed
2013	Li	Hepatic	Mouse	Post-injury induction (Treatment)	Yes	Not Described	Mixed
2013	Roccaro	Cancer	Mouse	At the same time as injury induction	No	Not Described	Mixed
2013	Bruno	Cancer	Mouse	Post-injury induction (Treatment)	No	Yes	Yes
2014	Chen	Lung	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2014	Feng	Angiogenesis (e.g. matrigel plug)	Mouse	At the same time as injury induction	No	No	Yes
2014	Tan	Hepatic	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2014	Du	Cancer	Mouse	At the same time as injury induction	No	Yes	Adverse effects
2014	Raisi	Nerve	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2014	Zou	Renal	Rat	Post-injury induction (Treatment)	Yes	Not Described	Yes
2014	Zhang	Renal	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2014	Choi	Lung	Mouse	Post-injury induction (Treatment)	No	Yes	Mixed
2014	Feng	Cardiac	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2014	Choi	Renal	Mouse	Post-injury induction (Treatment)	Yes	Not Described	Yes
2014	Zhang	Transplantation	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2014	Bian	Cardiac	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2014	Rahman	Autoimmune, Diabetes	Mouse	Post-injury induction (Treatment)	No	Not Described	Adverse effects
2014	Zhu	Lung	Mouse	Post-injury induction (Treatment), At the same time as injury induction	No	Not Described	Yes
2015	Koch	Renal, Transplantation	Rat	Post-injury induction (Treatment)	No	Not Described	Mixed
2015	Zhao	Cardiac	Rat	Post-injury induction (Treatment)	No	Not Described	Mixed
2015	Kang	Cardiac	Rat	Post-injury induction (Treatment)	No	Yes	Yes

2015	Li	Lung	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2015	Yang	Neurological	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2015	Teng	Cardiac	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2015	Lou	Cancer	Mouse	Post-injury induction (Treatment)	No	Yes	Mixed
2015	Yang	Intestinal	Rat	Post-injury induction (Treatment)	Yes	Yes	Yes
2015	Geiger	Diabetes, Wound	Mouse	Post-injury induction (Treatment)	No	Yes	Yes
2015	Ti	Diabetes, Wound	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2015	Cruz	Lung	Mouse	Post-injury induction (Treatment)	No	Yes	Yes
2015	Wang	Sepsis	Mouse	Post-injury induction (Treatment)	Yes	Not Described	Yes
2015	Doepfner	Neurological	Mouse	Post-injury induction (Treatment)	No	Yes	Mixed
2015	Hu	Ischemia (e.g. testing angiogenesis)	Mouse	Post-injury induction (Treatment)	No	Yes	Yes
2015	Monsel	Lung	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2015	Wang	Renal	Mouse	Post-injury induction (Treatment)	No	Yes	Yes
2015	Choi	Renal	Mouse	Post-injury induction (Treatment)	Yes	Yes	Yes
2015	Nakamura	Muscle	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2015	Zhang	Wound, Angiogenesis (e.g. matrigel plug)	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2015	Ju	Renal	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2015	Zhang	Wound	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2015	Yu	Cardiac	Rat	Post-injury induction (Treatment)	Yes	Not Described	Yes
2015	Zhang	Wound	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2015	Ko	Cancer	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2015	Zhang	Neurological	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2015	Collino	Renal	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2016	Yu	Ophthalmic	Mouse	Post-injury induction (Treatment)	Yes	Not Described	Yes
2016	Tamura	Hepatic	Mouse	Post-injury induction (Treatment)	Yes	Not Described	Yes
2016	Shen	Renal	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2016	Zou	Renal	Rat	Post-injury induction (Treatment)	Yes	Not Described	Yes
2016	Gu	Renal	Rat	Post-injury induction (Treatment)	Yes	Not Described	Yes
2016	Nagaishi	Renal, Diabetes	Rat	Post-injury induction (Treatment)	Yes	Not Described	Yes
2016	Hu	Wound	Mouse	Post-injury induction (Treatment)	Yes	Yes	Yes
2016	Lee	Neurological	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2016	Xie	Bone/Joint	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2016	Zhang	Bone/Joint	Rat	Post-injury induction (Treatment)	No	Yes	Mixed
2016	Yin	Pancreas	Mouse, Rat	Post-injury induction (Treatment)	Yes	Yes	Yes
2016	Kalimuthu	Cancer	Mouse	Post-injury induction (Treatment)	Yes	Yes	Yes
2016	Chen	Neurological	Rat	Post-injury induction (Treatment)	Yes	Not Described	Yes
2016	Nakano	Neurological, Diabetes	Mouse	Post-injury induction (Treatment)	Yes	Not Described	Yes
2016	Wen	Transplantation, Diabetes	Mouse	Post-injury induction (Treatment)	Yes	Yes	Yes
2016	Liu	Neurological	Mouse	Post-injury induction (Treatment)	Yes	Not Described	Yes
2016	Zhang	Wound	Rat	Post-injury induction (Treatment)	No	Not Described	Mixed
2016	Zou	Renal	Rat	Post-injury induction (Treatment)	Yes	Not Described	Yes
2016	Wen	Irradiation	Mouse	Post-injury induction (Treatment)	Yes	Not Described	Yes
2016	Guo	Bone/Joint	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2016	Nong	Hepatic	Rat	Post-injury induction (Treatment)	No	Not Described	Yes

2016	Furuta	Bone/Joint	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2016	Hu	Neurological	Rat	Post-injury induction (Treatment)	No	Yes	Mixed
2016	Vrijssen	Angiogenesis (e.g. matrigel plug)	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2016	Li	Wound	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2016	Fang	Wound	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2016	Qi	Bone/Joint	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2016	Ophelders	Neurological	Sheep	Post-injury induction (Treatment)	No	Yes	Mixed
2016	Lin	Renal	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2016	Rager	Intestinal, Infection (non sepsis)	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2016	Aliotta	Lung	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2016	Qin	Bone/Joint	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2016	Zhang	Renal	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2016	Sun	Cardiac	Rat	Post-injury induction (Treatment)	No	Not Described	No benefits
2016	Kim	Neurological	Mouse	Post-injury induction (Treatment)	No	Not Described	Mixed
2016	Liu		Mouse	Post-injury induction (Treatment)	Yes	Not Described	Yes
2016	Wang	Graft <i>versus</i> host disease	Mouse	Pre-injury induction (Prevention)	No	Not Described	Yes
2016	Ti	Diabetes, Wound	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2016	Shi	Cancer	Mouse	Post-injury induction (Treatment)	No	Yes	Adverse effects
2016	Lange	Irradiation	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2017	Fujii	Graft <i>versus</i> host disease	Mouse	Post-injury induction (Treatment)	No	Yes	Yes
2017	Cheng	Intervertebral disc degeneration	Rat	Post-injury induction (Treatment)	No	Yes	Mixed
2017	Willis	Lung	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2017	Zhu	Cardiac	Mouse	Post-injury induction (Treatment)	No	Yes	Yes
2017	Webb	Neurological	Mouse	Post-injury induction (Treatment)	Yes	Yes	No benefits
2017	Wang	Wound	Mouse	Post-injury induction (Treatment)	Yes	Yes	Yes
2017	Mead	Ophthalmic	Rat	Post-injury induction (Treatment)	Yes	Not Described	Yes
2017	Yuan	Renal	Rat	Pre-injury induction (Prevention)	No	Not Described	Yes
2017	Soundara Rajan	Autoimmune	Mouse	Post-injury induction (Treatment)	No	Yes	Yes
2017	El-Tookhy	Wound	Dog	Post-injury induction (Treatment)	No	Yes	Yes
2017	Schoefinius	Total Body Irradiation	Mouse	Post-injury induction (Treatment)	Yes	Not Described	Mixed
2017	Huang	Spinal cord injury	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2017	Chen	Renal	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2017	Zhang	Neurological	Rat	Post-injury induction (Treatment)	No	Yes	Mixed
2017	Stone	Lung	Mouse	Pre-injury induction (Prevention)	No	Yes	Yes
2017	Horn	Neurological	Rat	Both Prevention and Treatment Protocols	No	Yes	Mixed
2017	Cosenza	Bone/Joint	Mouse	Post-injury induction (Treatment)	No	Not Described	Mixed
2017	Shi	Diabetes, Wound	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2017	Rajendran	Hair growth	Mouse	Post-injury induction (Treatment)	Yes	Not Described	Yes
2017	Bruno	Renal	Mouse	Post-injury induction (Treatment)	No	Not Described	Mixed
2017	Aliotta	Lung	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2017	Komaki	Ischemia (e.g. testing angiogenesis)	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes

2017	Li	Neurological	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2017	Gangadaran	Ischemia (e.g. testing angiogenesis)	Mouse	Post-injury induction (Treatment)	Yes	Yes	Yes
2017	Deng	Neurological	Mouse	Pre-injury induction (Prevention)	No	Yes	Mixed
2017	Bai	Autoimmune, Ophthalmic	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2017	Sun	Hepatic	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2017	Gong	Angiogenesis (e.g. matrigel plug)	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2017	Qu	Hepatic	Mouse	Post-injury induction (Treatment)	No	Yes	Yes
2017	Casado	Bone/Joint	Pig	Post-injury induction (Treatment)	No	Not Described	Mixed
2017	Xie	Bone/Joint	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2017	Otero-Ortega	Neurological	Rat	Post-injury induction (Treatment)	Yes	Yes	Mixed
2017	Wang	Cardiac	Mouse	Post-injury induction (Treatment)	No	Not Described	Mixed
2017	Haga	Hepatic	Mouse	Post-injury induction (Treatment)	Yes	Not Described	Yes
2017	Ma	Cardiac	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2017	Wang	Bone/Joint	Mouse	Post-injury induction (Treatment)	No	Yes	Mixed
2017	Long	Neurological	Mouse	Post-injury induction (Treatment)	Yes	Yes	Yes
2017	Li	Bone/Joint	Rabbit	Post-injury induction (Treatment)	No	Yes	Yes
2017	Eirin	Renal	Pig	Post-injury induction (Treatment)	Yes	Yes	Yes
2017	de Castro	Lung	Mouse	Post-injury induction (Treatment)	No	Yes	Mixed
2017	Tang	Lung	Mouse	At the same time as injury induction	No	Not Described	Yes
2017	Gonzalez-King	Angiogenesis (e.g. matrigel plug)	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2017	Haga	Hepatic	Mouse	Pre-injury induction (Prevention)	Yes	Not Described	Yes
2017	Mao	Intestinal	Mouse	Post-injury induction (Treatment)	Yes	Yes	Yes
2017	Shigemoto-Kuroda	Autoimmune, Ophthalmic, Diabetes	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2017	Du	Ischemia (e.g. testing angiogenesis)	Mouse	Post-injury induction (Treatment)	No	Yes	Yes
2017	Chen	Hepatic	Mouse	Pre-injury induction (Prevention)	Yes	Not Described	Yes
2017	Song	Sepsis	Mouse	Post-injury induction (Treatment)	Yes	Yes	Yes
2017	Moisseiev	Ophthalmic	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2017	Liu	Cardiac	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2017	Zhu	Bone/Joint	Mouse	Post-injury induction (Treatment)	No	Yes	Yes
2017	Xin	Neurological	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2017	Yan	Hepatic	Mouse	Post-injury induction (Treatment)	Yes	Yes	Yes
2017	Tao	Bone/Joint	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2017	Liu	Bone/Joint	Rat	Pre-injury induction (Prevention)	No	Yes	Yes
2017	Kamerkar	Cancer	Mouse	Post-injury induction (Treatment)	Yes	Yes	Mixed
2017	Du	Hepatic	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2017	Wang	Renal	Rat	Pre-injury induction (Prevention)	No	Not Described	Yes
2017	Zhao	Wound	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2017	Shao	Cardiac	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2017	Tao	Diabetes, Wound	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2017	Huang	Transplantation	Mouse	At the same time as injury induction	No	Yes	Yes
2017	Tamura	Hepatic	Mouse	Post-injury induction (Treatment)	Yes	Not Described	Yes

2017	Grigorian-Shamagian	Cancer	Mouse	Post-injury induction (Treatment)	No	Yes	No benefits
2017	Ranghino	Renal	Mouse	Post-injury induction (Treatment)	Yes	Not Described	Yes
2017	Cui	Cardiac	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2017	Henao Agudelo	Intestinal, Infection (non sepsis)	Mouse	Post-injury induction (Treatment)	No	Not Described	Mixed
2017	Xin	Neurological	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2017	Lo Sicco	Muscle, Angiogenesis (e.g. matrigel plug)	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2017	de Almeida	Renal	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2018	Luther	Cardiac	Mouse	Pre-injury induction (Prevention)	No	Not Described	Yes
2018	Williams	Neurological, Shock	Pig	Post-injury induction (Treatment)	No	Yes	Yes
2018	McCulloh	Intestinal, Infection (non sepsis)	Rat	Pre-injury induction (Prevention)	No	Yes	Yes
2018	Zhang	Graft <i>versus</i> host disease	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2018	Barile	Cardiac	Rat	Post-injury induction (Treatment)	No	Not Described	Mixed
2018	Otero-Ortega	Neurological	Rat	Post-injury induction (Treatment)	Yes	Yes	Mixed
2018	Ahn	Lung	Rat	Post-injury induction (Treatment)	Yes	Yes	Yes
2018	Jiang	Hepatic	Mouse	Post-injury induction (Treatment)	Yes	Yes	Mixed
2018	Diomedede	Bone/Joint	Rat	Post-injury induction (Treatment)	Yes	Yes	Yes
2018	He	Ophthalmic	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2018	Blazquez	Incisional hernia	Mouse	Post-injury induction (Treatment)	No	Not Described	Mixed
2018	Shen	Neurological	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2018	Lang	Cancer	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2018	Wang	Cardiac	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2018	Cui	Neurological	Mouse	Post-injury induction (Treatment)	Yes	Not Described	Yes
2018	Damania	Hepatic	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2018	Li	Bone/Joint	Mouse	Post-injury induction (Treatment)	Yes	Yes	Yes
2018	Zhu	Diabetes, Erectile dysfunction	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2018	Khatri	Lung	Pig	Post-injury induction (Treatment)	No	Not Described	Yes
2018	Mead	Ophthalmic	Rat	Post-injury induction (Treatment)	No	Not Described	Mixed
2018	Wang	Neurological	Mouse	Post-injury induction (Treatment)	No	Yes	Yes
2018	Otsuru	Bone/Joint	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2018	Wu	Renal	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2018	Potter	Lung, Shock	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2018	Ruppert	Spinal cord injury	Rat	Post-injury induction (Treatment)	No	Yes	Yes
2018	Han	Transplantation	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2018	Chen	Hepatic	Mouse	Post-injury induction (Treatment)	No	Yes	Yes
2018	Mardpour	Hepatic	Rat	Post-injury induction (Treatment)	Yes	Not Described	Yes
2018	O'Brien	Cancer	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes
2018	Chang	Sepsis	Rat	Post-injury induction (Treatment)	No	Not Described	Yes
2018	Tian	Bone/Joint	Rabbit	Post-injury induction (Treatment)	No	Yes	Yes
2018	Kou	Diabetes, Wound	Mouse	Post-injury induction (Treatment)	No	Yes	Yes
2018	Chen	Diabetes, Wound	Mouse	Post-injury induction (Treatment)	No	Yes	Yes
2018	Jia	Renal	Rat	Pre-injury induction (Prevention)	No	Yes	Yes
2018	Cosenza	Bone/Joint	Mouse	Post-injury induction (Treatment)	No	Not Described	Yes

Supplemental File B4 – Dosage Data

<u>Year</u>	<u>First Author's Last Name</u>	<u>Single or Multiple Dose Study</u>	<u>Dose</u>	<u>Dosage Units</u>	<u>Route of Administration</u>	<u>No. of Doses</u>	<u>If multi-dose study, how often were treatments given?</u>	<u>Was a dose-response study conducted?</u>	<u>If a dose-response study was conducted, how many different doses were administered?</u>	<u>If a dose-response study was conducted, what was the dose range?</u>
2009	Bruno	Single Dose of EVs	15ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No		
2010	Lai	Single Dose of EVs	10ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No		
2011	Chen	Single Dose of EVs	0.3ug	EV protein concentration (e.g. ug)	IV - Other (e.g. peripheral sites)	1		No		
2011	Gatti	Single Dose of EVs	30ug	EV protein concentration (e.g. ug)	IV - Other (e.g. peripheral sites)	1		No		
2012	Lai	Single Dose of EVs	0.4ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No		
2012	Lee	Single Dose of EVs, Both (Same experiment)	0.1ug	EV protein concentration (e.g. ug)	IV - jugular vein, IV - tail vein	2	Day 0 and 4	Yes	2	0.1 and 10 ug
2012	Reis	Single Dose of EVs	100ug/mL	EV protein concentration (e.g. ug)	IV - tail vein	1		No		
2012	Zhang	Multiple Doses of EV	10, 50 and 100 ug	EV protein concentration (e.g. ug)	Intramuscular (IM)	1		Yes	3	10, 50 and 100 ug
2012	van Koppen	Multiple Doses of EV	7ug	EV protein concentration (e.g. ug)	IV - tail vein	8	Twice daily for 4 days	No		
2012	Bruno	Both (Same experiment)	100ug (+50ug for subsequent doses)	EV protein concentration (e.g. ug)	IV - tail vein	6	8h and days 2, 6, 10, 14, and 18	No		
2012	He	Multiple Doses of EV	30ug	EV protein concentration (e.g. ug)	IV - tail vein	3	Days 2, 3, 5	No		
2013	Kilpinen	Single Dose of EVs	Not Described	Not Described	Intra-arterial	1		No		
2013	Lee	Single Dose of EVs	100ug or 200ug	EV protein concentration (e.g. ug)	Subcutaneous	1		Yes	2	100 or 200 ug
2013	Xin	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No		
2013	Zhou	Single Dose of EVs	200ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		No		
2013	Wu	Single Dose of EVs	200ug	EV protein concentration (e.g. ug)	Subcutaneous	1		No		
2013	Katakowski	Single Dose of EVs	50ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		No		
2013	Arslan	Single Dose of EVs	1, 4 or 16ug/kg	EV protein / weight of animal (e.g. ug/kg)	IV - tail vein	1		Yes	3	1, 4 or 16 ug/kg
2013	Li	Single Dose of EVs	250ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		No		
2013	Roccaro	Multiple Doses of EV	1ug	EV protein concentration (e.g. ug)	Implanted (e.g. with scaffolds)	2, 3, 4	Ever 4 days for 7, 10 or 14 days	No		
2013	Bruno	Multiple Doses of EV	100ug (first dose),	EV protein concentration (e.g. ug)	Direct tissue injection	3, 4	Weekly for 3 or 4 weeks	No		

			50ug (subsequen t)						
2014	Chen	Multiple Doses of EV	30ug	EV protein concentration (e.g. ug)	IV - tail vein	14	Once every 2 days for 2 weeks	No	
2014	Feng	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	Subcutaneous	1		No	
2014	Tan	Single Dose of EVs	0.4ug	EV protein concentration (e.g. ug)	Intrasplenic	1		No	
2014	Du	Single Dose of EVs	200ug/mL	EV protein concentration (e.g. ug)	Subcutaneous	1		No	
2014	Raisi	Single Dose of EVs	45ug	EV protein concentration (e.g. ug)	Implanted (e.g. with scaffolds)	1		No	
2014	Zou	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No	
2014	Zhang	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No	
2014	Choi	Multiple Doses of EV	10ug	EV protein concentration (e.g. ug)	IV - tail vein	2	12 and 14 weeks	No	
2014	Feng	Single Dose of EVs	1ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		No	
2014	Choi	Single Dose of EVs	2e7 particles	EV particle concentration (e.g. exo's/mL PBS)	IV - tail vein	1		No	
2014	Zhang	Both (Separate experiments)	0.3ug	EV protein concentration (e.g. ug)	Subcutaneous, Intrasplenic	11	Daily for 4 days, then every other day for 15 days	No	
2014	Bian	Single Dose of EVs	80ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		No	
2014	Rahman	Multiple Doses of EV	2.5ug (first dose) and 5ug (2 doses)	EV protein concentration (e.g. ug) # of EVs made over time by cell culture (MSCs)	Intraperitoneal	3	Once per week	No	
2014	Zhu	Single Dose of EVs	3e6 MSC Equivalent Not Described		IV - jugular vein, Intratracheal	1		Yes	2 3e6 or 6e6 MSC Equivalent
2015	Koch	Single Dose of EVs	Not Described	Not Described	Not described	1		No	
2015	Zhao	Single Dose of EVs	400ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No	
2015	Kang	Single Dose of EVs	Not Described	Not Described	Implanted (e.g. with scaffolds)	1		No	
2015	Li	Single Dose of EVs	1.5ug/g	EV protein / weight of animal (e.g. ug/kg)	IV - tail vein	1		Yes	2 1.5 or 3ug/g
2015	Yang	Single Dose of EVs	400ug/kg	EV protein / weight of animal (e.g. ug/kg)	Not described	1		No	
2015	Teng	Single Dose of EVs	80ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		No	
2015	Lou	Single Dose of EVs	50ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		No	
2015	Yang	Single Dose of EVs	50, 100 or 200 ug	EV protein concentration (e.g. ug)	IV - tail vein	1		Yes	3 50, 100 and 200 ug
2015	Geiger	Single Dose of EVs	5 or 50 ug	EV protein concentration (e.g. ug)	Direct tissue injection, Subcutaneous	1		Yes	2 5 or 50 ug

2015	Ti	Single Dose of EVs	60ug	EV protein concentration (e.g. ug)	Subcutaneous	1		No
2015	Cruz	Single Dose of EVs	3e6 MSC Equivalent	EV dose based on cell count (e.g. per weight of animal)	IV - tail vein	1		No
2015	Wang	Single Dose of EVs	2ug/g	EV protein / weight of animal (e.g. ug/kg)	IV - tail vein	1		No
2015	Doepfner	Both (Separate experiments)	2e6 MSC Equivalent	EV dose based on cell count (e.g. per weight of animal)	IV - Other (e.g. peripheral sites)	3	Day 1, 3 and 5	No
2015	Hu	Multiple Doses of EV	200ug	EV protein concentration (e.g. ug)	Intramuscular (IM)	4	Day 0	No
2015	Monsel	Single Dose of EVs	3e6, 6e6 or 9e6 MSC Equivalent	# of EVs made over time by cell culture (MSCs)	IV - Other (e.g. peripheral sites), Intratracheal	1		Yes 3 3e6, 6e6 or 9e6 MSC Equivalent
2015	Wang	Single Dose of EVs	30ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No
2015	Choi	Single Dose of EVs	2e7 particles	EV particle concentration (e.g. exo's/mL PBS)	IV - tail vein	1		No
2015	Nakamura	Multiple Doses of EV	Not Described	Not Described	Intramuscular (IM)	3	Day 1, 3 and 5	No
2015	Zhang	Single Dose of EVs	200ug	EV protein concentration (e.g. ug)	Subcutaneous	1		No
2015	Ju	Single Dose of EVs	30ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No
2015	Zhang	Single Dose of EVs	160ug	EV protein concentration (e.g. ug)	Subcutaneous	1		No
2015	Yu	Single Dose of EVs	4e6 MSC Equivalent	EV dose based on cell count (e.g. per weight of animal)	Direct tissue injection	1		No
2015	Zhang	Single Dose of EVs	200ug	EV protein concentration (e.g. ug)	Subcutaneous	1		No
2015	Ko	Multiple Doses of EV	2000ug	EV protein concentration (e.g. ug)	IV - Other (e.g. peripheral sites)	2	Day 10 and 20	Yes 3 1000, 2000and 4000 ug
2015	Zhang	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No
2015	Collino	Single Dose of EVs	2.2e8	EV particle concentration (e.g. exo's/mL PBS)	IV - tail vein	1		No
2016	Yu	Single Dose of EVs	2.5ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		Yes 6 0.05-10ug
2016	Tamura	Both (Same experiment)	10ug	EV protein concentration (e.g. ug)	IV - Other (e.g. peripheral sites)	3	0, 8 or 16h	No
2016	Shen	Single Dose of EVs	200ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		No
2016	Zou	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No
2016	Gu	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No
2016	Nagaishi	Single Dose of EVs	5.3e7	EV particle concentration (e.g. exo's/mL PBS)	Direct tissue injection	1		No
2016	Hu	Single Dose of EVs	200ug	EV protein concentration (e.g. ug)	IV - tail vein, Subcutaneous	1		No

2016	Lee	Single Dose of EVs	0.2mg/kg	EV protein / weight of animal (e.g. ug/kg)	Intra-arterial	1		No
2016	Xie	Single Dose of EVs	20ug	EV protein concentration (e.g. ug)	Subcutaneous	1		No
2016	Zhang	Multiple Doses of EV	100ug	EV protein concentration (e.g. ug)	Direct tissue injection	6, 12	Weekly for 6 or 12 weeks	No
2016	Yin	Single Dose of EVs	100 or 1000 ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No
2016	Kalimuthu	Multiple Doses of EV	50ug	EV protein concentration (e.g. ug)	Direct tissue injection	2	Day 21 and 26	No
2016	Chen	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - Other (e.g. peripheral sites)	1		No
2016	Nakano	Multiple Doses of EV	0.5ug	EV protein concentration (e.g. ug)	Intracerebroventricle	5	Daily for 5 days	No
2016	Wen	Multiple Doses of EV	10mg/kg	EV protein / weight of animal (e.g. ug/kg)	IV - Other (e.g. peripheral sites)	4	Every 2 days for 4 doses	No
2016	Liu	Multiple Doses of EV	27ug	EV protein concentration (e.g. ug)	IV - jugular vein	2	Day 6 and 9	No
2016	Zhang	Single Dose of EVs	200ug	EV protein concentration (e.g. ug)	Subcutaneous	1		No
2016	Zou	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No
2016	Wen	Multiple Doses of EV	4e9 particles	EV particle concentration (e.g. exo's/mL PBS)	IV - tail vein	3	6, 24, 72h	No
2016	Guo	Multiple Doses of EV	1e11 particles	EV particle concentration (e.g. exo's/mL PBS)	IV - tail vein	9	First 3 days for 1 or 3 weeks	No
2016	Nong	Single Dose of EVs	600ug	EV protein concentration (e.g. ug)	IV - Other (e.g. peripheral sites)	1		No
2016	Furuta	Multiple Doses of EV	1e6 MSC Equivalent	EV dose based on cell count (e.g. per weight of animal)	Direct tissue injection	2	Days 1 and 8	No
2016	Hu	Multiple Doses of EV	264e4 particles	EV particle concentration (e.g. exo's/mL PBS)	IV - Other (e.g. peripheral sites)	3	Day 1, 3 and 5	No
2016	Vrijssen	Single Dose of EVs	20ug	EV protein concentration (e.g. ug)	Subcutaneous	1		Yes 2 1, 2 or 4 ug
2016	Li	Single Dose of EVs	800ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No
2016	Fang	Single Dose of EVs	Not Described	EV protein concentration (e.g. ug)	Subcutaneous	1		No
2016	Qi	Single Dose of EVs	100 or 200 ug	EV protein concentration (e.g. ug)	Implanted (e.g. with scaffolds)	1		Yes 2 100 or 200 ug
2016	Ophelders	Multiple Doses of EV	2e7 MSC Equivalent	EV dose based on cell count (e.g. per weight of animal)	Intra-arterial	2	1h and 4 days	No
2016	Lin	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - Other (e.g. peripheral sites)	1		No
2016	Rager	Single Dose of EVs	2.5e9 particles	EV particle concentration (e.g. exo's/mL PBS)	Intraperitoneal	1		No
2016	Aliotta	Multiple Doses of EV	25ug	EV protein concentration (e.g. ug)	IV - tail vein	3, 4	3 doses (daily), or 4 doses (1/week)	No

2016	Qin	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	Implanted (e.g. with scaffolds)	1		No		
2016	Zhang	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No		
2016	Sun	Multiple Doses of EV	400ug or 800ug	EV protein concentration (e.g. ug)	IV - tail vein	7	Daily for 7 days	Yes	2	400 and 800 ug
2016	Kim	Single Dose of EVs	3.8-30ug	EV protein concentration (e.g. ug)	IV - tail vein	1		Yes	4	3.8-30ug
2016	Liu	Multiple Doses of EV	20ug	EV protein concentration (e.g. ug)	Intraperitoneal	11	Every other day for 20 days	No		
2016	Wang	Multiple Doses of EV	200ug	EV protein concentration (e.g. ug)	IV - tail vein	2	Day 0 and 7	No		
2016	Ti	Multiple Doses of EV	60ug	EV protein concentration (e.g. ug)	Subcutaneous	10	Daily for 10 days	No		
2016	Shi	Multiple Doses of EV	Not Described	Not Described	Direct tissue injection	7	Every 3 days until day 20	No		
2016	Lange	Not Described	Not Described	Not Described	Not described	1		No		
2017	Fujii	Single Dose of EVs	2e6 MSC equivalent per kg	EV dose based on cell count (e.g. per weight of animal)	IV - tail vein	1		No		
2017	Cheng	Multiple Doses of EV	1.5e6	EV particle concentration (e.g. exo's/mL PBS)	Direct tissue injection	2	At 1 week and 5 weeks after puncture	No		
2017	Willis	Single Dose of EVs	0.5e6 MSC over 36h equivalent	# of EVs made over time by cell culture (MSCs)	IV - Other (e.g. peripheral sites)	1		No		
2017	Zhu	Single Dose of EVs	2e7 MSC Equivalent	# of EVs made over time by cell culture (MSCs)	Direct tissue injection	1		No		
2017	Webb	Multiple Doses of EV	2.7e11 particles/kg	EV particles / weight of animal (e.g. exo's/kg)	IV - tail vein	3	At 2, 14, and 38h post injury	No		
2017	Wang	Single Dose of EVs	200ug	EV protein concentration (e.g. ug)	IV - Other (e.g. peripheral sites)	1		No		
2017	Mead	Multiple Doses of EV	3e9 particles	EV particle concentration (e.g. exo's/mL PBS)	Direct tissue injection	3	Day 0, 7 and 14	No		
2017	Yuan	Single Dose of EVs	1e12 particles	EV particle concentration (e.g. exo's/mL PBS)	IV - Other (e.g. peripheral sites)	1		No		
2017	Soundara Rajan	Single Dose of EVs	24ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No		
2017	El-Tookhy	Single Dose of EVs	2e6 MSC equivalent	EV dose based on cell count (e.g. per weight of animal)	Subcutaneous	1		No		
2017	Schoefinius	Single Dose of EVs	1e6 MSC Equivalent	EV dose based on cell count (e.g. per weight of animal)	IV - tail vein	1		No		
2017	Huang	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No		
2017	Chen	Single Dose of EVs	100 ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No		

2017	Zhang	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No	
2017	Stone	Single Dose of EVs	1e6	EV particle concentration (e.g. exo's/mL PBS)	Intratracheal	1		No	
2017	Horn	Multiple Doses of EV	1e8 MSC Equivalent per kg	EV dose based on cell count (e.g. per weight of animal)	Intraperitoneal	2	3h before and 24h after injury	No	
2017	Cosenza	Single Dose of EVs	0.5ug MVs or 0.25ug Exos	EV protein concentration (e.g. ug)	Direct tissue injection	1		No	
2017	Shi	Multiple Doses of EV	150ug	EV protein concentration (e.g. ug)	Implanted (e.g. with scaffolds)	3, 6	Every 3 days for 1 week or 2 weeks	No	
2017	Rajendran	Multiple Doses of EV	200ug	EV protein concentration (e.g. ug)	Subcutaneous	15	Every 2 days for 28days	No	
2017	Bruno	Single Dose of EVs	1.65e8	EV particle concentration (e.g. exo's/mL PBS)	IV - Other (e.g. peripheral sites)	1		No	
2017	Aliotta	Multiple Doses of EV	25ug	EV protein concentration (e.g. ug)	IV - tail vein	3	Daily for 3 days	No	
2017	Komaki	Multiple Doses of EV	2.2ug/dose	EV protein concentration (e.g. ug)	Subcutaneous	2	Daily for 2 days	No	
2017	Li	Not Described	1000ug/m L	EV protein concentration (e.g. ug)	Intracerebral	1		Yes	100 to 1000 ug/mL
2017	Gangadaran	Multiple Doses of EV	100ug	EV protein concentration (e.g. ug)	Intramuscular (IM)	6	twice a week for 3 weeks	No	
2017	Deng	Single Dose of EVs	200ug	EV protein concentration (e.g. ug)	Intracerebroventricle	1		No	
2017	Bai	Multiple Doses of EV	50ug	EV protein concentration (e.g. ug)	Direct tissue injection	7	Daily for 7 days	Yes	4 10-100 ug
2017	Sun	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - Other (e.g. peripheral sites)	1		No	
2017	Gong	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	Subcutaneous	1		No	
2017	Qu	Multiple Doses of EV	40ug	EV protein concentration (e.g. ug)	Intrasplenic	16	Twice per week for 8 weeks	No	
2017	Casado	Single Dose of EVs	500ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		No	
2017	Xie	Single Dose of EVs	20ug	EV protein concentration (e.g. ug)	Implanted (e.g. with scaffolds)	1		No	
2017	Otero-Ortega	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No	
2017	Wang	Single Dose of EVs	Not Described	Not Described	IV - tail vein	1		No	
2017	Haga	Single Dose of EVs	2e8 to 2e10	EV particle concentration (e.g. exo's/mL PBS)	IV - Other (e.g. peripheral sites), Intraperitoneal	1		Yes	2e9 or 2e10 particles, IP (Fig 3D)
2017	Ma	Single Dose of EVs	400ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No	
2017	Wang	Multiple Doses of EV	Not Described	EV protein concentration (e.g. ug)	Direct tissue injection	10	Every 3 days for 4 weeks	No	
2017	Long	Multiple Doses of EV	15ug per dose	EV protein concentration (e.g. ug)	IV - tail vein, Intranasal	2	2 doses in 18h	No	
2017	Li	Single Dose of EVs	40ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		No	

2017	Eirin	Single Dose of EVs	Not Described	EV particle concentration (e.g. exo's/mL PBS)	Direct tissue injection	1		No	
2017	de Castro	Single Dose of EVs	1e5 MSC Equivalent	EV protein concentration (e.g. ug)	IV - jugular vein	1		No	
2017	Tang	Single Dose of EVs	Not Described	# of EVs made over time by cell culture (MSCs)	Intratracheal	1		No	
2017	Gonzalez-King	Single Dose of EVs	1.5ug	EV protein concentration (e.g. ug)	Subcutaneous	1		No	
2017	Haga	Single Dose of EVs	2e10	EV particle concentration (e.g. exo's/mL PBS)	IV - tail vein	1		No	
2017	Mao	Multiple Doses of EV	400ug	EV protein concentration (e.g. ug)	IV - tail vein	3	Days 3, 6 and 9	No	
2017	Shigemoto-Kuroda	Both (Separate experiments)	3ug, or 30ug	EV protein concentration (e.g. ug)	IV - tail vein	2	Day 0 and 4	Yes	2
2017	Du	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	Intramuscular (IM)	1		No	3 or 30 ug (Fig 1)
2017	Chen	Single Dose of EVs	Not Described	EV protein concentration (e.g. ug)	IV - tail vein	1		No	
2017	Song	Single Dose of EVs	30ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No	
2017	Moisseiev	Single Dose of EVs	20ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		No	
2017	Liu	Single Dose of EVs	5ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		No	
2017	Zhu	Multiple Doses of EV	8e7 particles	EV particle concentration (e.g. exo's/mL PBS)	Direct tissue injection	3	Days 7, 14, 21	No	
2017	Xin	Single Dose of EVs	3e11 particles	EV particle concentration (e.g. exo's/mL PBS)	Intra-arterial	1		No	
2017	Yan	Single Dose of EVs	32mg/kg	EV protein / weight of animal (e.g. ug/kg)	IV - tail vein, Oral Gavage	1		Yes	3
2017	Tao	Multiple Doses of EV	1e10	EV particle concentration (e.g. exo's/mL PBS)	Direct tissue injection	4	Once a week for 4 weeks	No	
2017	Liu	Multiple Doses of EV	1e9 or 1e10 particles	EV particle concentration (e.g. exo's/mL PBS)	IV - tail vein	9	3 per week for 3 weeks	Yes	2
2017	Kamerkar	Multiple Doses of EV	1e8 particles	EV particle concentration (e.g. exo's/mL PBS)	Intraperitoneal	1	Dosed every other day	No	
2017	Du	Single Dose of EVs	2.5e12	EV particle concentration (e.g. exo's/mL PBS)	IV - Other (e.g. peripheral sites)	1		No	
2017	Wang	Single Dose of EVs	200ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		No	
2017	Zhao	Single Dose of EVs	2.5, 5 or 10 ug	EV protein concentration (e.g. ug)	Subcutaneous	1		Yes	3
2017	Shao	Single Dose of EVs	20ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		No	2.5, 5 or 10 ug
2017	Tao	Single Dose of EVs	Not Described	Not Described	Subcutaneous	1		No	

2017	Huang	Single Dose of EVs	50 or 100ug	EV protein concentration (e.g. ug)	Subcutaneous	1		Yes	2	50 or 100 ug
2017	Tamura	Single Dose of EVs	10ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No		
2017	Grigorian-Shamagian	Multiple Doses of EV	1000ug	EV protein concentration (e.g. ug) # of EVs made over time by cell culture (MSCs)	Intraperitoneal	2	Day 0 and 2 weeks;	No		
2017	Ranghino	Single Dose of EVs	4e8 particles	EV protein concentration (e.g. ug)	IV - tail vein	1		No		
2017	Cui	Single Dose of EVs	400ug	EV particle concentration (e.g. exo's/mL PBS)	IV - tail vein	1		No		
2017	Henao Agudelo	Multiple Doses of EV	8.38e9	EV protein concentration (e.g. ug)	Intraperitoneal	4	0, 24, 48 and 72h	No		
2017	Xin	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - Other (e.g. peripheral sites)	1		No		
2017	Lo Sicco	Both (Separate experiments)	5ug (matrigel) or 1ug (direct, 2 doses)	EV protein concentration (e.g. ug)	Intramuscular (IM)	2	2h and day 4	No		
2017	de Almeida	Multiple Doses of EV	100ug	EV protein concentration (e.g. ug)	IV - Other (e.g. peripheral sites)	2	Days 1 and 3	No		
2018	Luther	Single Dose of EVs	0.5ug/g	EV protein / weight of animal (e.g. ug/kg)	Direct tissue injection	1		No		
2018	Williams	Multiple Doses of EV	1e13 particles	EV particle concentration (e.g. exo's/mL PBS)	IV - Other (e.g. peripheral sites)	5	9h post injury, and post-injury days 1, 5, 9, 13	No		
2018	McCulloh	Single Dose of EVs	4e8	EV particle concentration (e.g. exo's/mL PBS)	Intraperitoneal	1		Yes	6	1.3e5 to 4e8 particles
2018	Zhang	Multiple Doses of EV	1 or 10 ug	EV protein concentration (e.g. ug)	Intraperitoneal	12	Every 3 days	Yes	2	1ug and 10ug
2018	Barile	Single Dose of EVs	10e11 particles	EV particle concentration (e.g. exo's/mL PBS)	Direct tissue injection	1		No		
2018	Otero-Ortega	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No		
2018	Ahn	Single Dose of EVs	20ug	EV protein concentration (e.g. ug)	Intratracheal	1		No		
2018	Jiang	Single Dose of EVs	Variable (6.4e9 particles to 2.4e11 particles/kg)	EV particle concentration (e.g. exo's/mL PBS), EV particles / weight of animal (e.g. exo's/kg)	IV - tail vein	1		Yes	3	6e10/kg to 2.4e11/kg
2018	Diomedede	Single Dose of EVs	100uL	Not Described	Implanted (e.g. with scaffolds)	1		No		
2018	He	Single Dose of EVs	0.05ug, 0.1ug or 0.15ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		Yes	3	0.05ug, 0.1ug or 0.15ug
2018	Blazquez	Single Dose of EVs	4000ug	EV protein concentration (e.g. ug)	Implanted (e.g. with scaffolds)	1		No		
2018	Shen	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No		

2018	Lang	Multiple Doses of EV	10e10 particles	EV particle concentration (e.g. exo's/mL PBS)	Intra-arterial, Intraperitoneal	Up to 56 doses	Every other day up to 110 days, also Day 14 and day 21	No	
2018	Wang	Single Dose of EVs	400ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No	
2018	Cui	Multiple Doses of EV	150ug	EV protein concentration (e.g. ug)	IV - Other (e.g. peripheral sites)	9	Bi-weekly for 4 months	No	
2018	Damania	Single Dose of EVs	50ug	EV protein concentration (e.g. ug)	IV - Other (e.g. peripheral sites)	1		No	
2018	Li	Single Dose of EVs	250ug	EV protein concentration (e.g. ug)	Implanted (e.g. with scaffolds)	1		No	
2018	Zhu	Single Dose of EVs	10 or 100 ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		Yes	2 10ug or 100ug
2018	Khatri	Single Dose of EVs	80ug/kg	EV protein / weight of animal (e.g. ug/kg)	Intratracheal	1		No	
2018	Mead	Both (Same experiment)	3e9 particles	EV particle concentration (e.g. exo's/mL PBS)	Direct tissue injection	2, 3, 8	single, weekly, monthly	No	
2018	Wang	Multiple Doses of EV	100ug	EV protein concentration (e.g. ug)	Intracerebroventricle	8	every 2 days for 2 weeks	No	
2018	Otsuru	Multiple Doses of EV	8.88e8 particles	EV particle concentration (e.g. exo's/mL PBS)	IV - tail vein	4	Once a week for 4 weeks	No	
2018	Wu	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - tail vein	1		No	
2018	Potter	Single Dose of EVs	30ug	EV protein concentration (e.g. ug)	Intra-arterial	1		No	
2018	Ruppert	Single Dose of EVs	1e9 particles	EV particle concentration (e.g. exo's/mL PBS)	IV - tail vein	1		No	
2018	Han	Multiple Doses of EV	50ug	EV protein concentration (e.g. ug)	Direct tissue injection	2, 4, 6, 8	every week for 2, 4, 6, or 8 weeks	No	
2018	Chen	Multiple Doses of EV	Not Described	Not Described	Intraperitoneal	3	once per week for 3 weeks	No	
2018	Mardpour	Single Dose of EVs	350ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		No	
2018	O'Brien	Multiple Doses of EV	2.6e7	EV particle concentration (e.g. exo's/mL PBS)	IV - tail vein	4	Days 0, 3, 6 and 9	No	
2018	Chang	Single Dose of EVs	100ug	EV protein concentration (e.g. ug)	IV - Other (e.g. peripheral sites)	1		No	
2018	Tian	Single Dose of EVs	10ug	EV protein concentration (e.g. ug)	Implanted (e.g. with scaffolds)	1		No	
2018	Kou	Single Dose of EVs	40ug or 200ug	EV protein concentration (e.g. ug)	Subcutaneous, Submucosal	1		No	
2018	Chen	Single Dose of EVs	200ug	EV protein concentration (e.g. ug)	Subcutaneous	1		No	
2018	Jia	Single Dose of EVs	200ug	EV protein concentration (e.g. ug)	Direct tissue injection	1		No	
2018	Cosenza	Multiple Doses of EV	0.25ug	EV protein concentration (e.g. ug)	IV - Other (e.g. peripheral sites)	2	Days 18 and 24	Yes	3 0.062, 0.125 and 0.25 ug

Supplemental File B5 – MSC-EV Modifications

<u>Year</u>	<u>Author Last Name</u>	<u>Disease Model/Injury</u>	<u>General Type of Modification</u>	<u>Specific Type of Modification</u>	<u>Biological Factor / Target</u>	<u>Specific Factor</u>	<u>Reduced or Enhanced Expression</u>	<u>Mechanism of Action #1</u>	<u>Mechanism of Action #2</u>	<u>Mechanism of Action #3</u>	<u>Mechanism of Action #4</u>	<u>Reason for modification</u>	<u>Did modified (enhanced) EVs outperform unmodified EVs?</u>	<u>Did modified (enhanced) EVs improve organ function?</u>	<u>Did modified (enhanced) EVs improve survival?</u>
2009a	Bruno	Kidney, Acute Kidney Injury	Modified (EVs directly)	EV preconditioning	Enzyme	RNAse	N/A	Apoptosis	Proliferation			Mechanism of action	N/A	N/A	N/A
2009b	Bruno	Kidney, Acute Kidney Injury	Modified (EVs directly)	EV preconditioning	Enzyme	Trypsin	N/A	EV incorporation into target cells				Mechanism of action	N/A	N/A	N/A
2009c	Bruno	Kidney, Acute Kidney Injury	Modified (EVs directly)	EV preconditioning	Chemical	sHA (soluble hyaluronic acid)	N/A	EV incorporation into target cells				Mechanism of action	N/A	N/A	N/A
2011	Chen	Heart, Myocardial infarction (ischemia-reperfusion)	Modified (MSCs before EV isolation)	MSC transduction	Protein	MYC	Overexpression	Immortal cell line				Enhancement	No comparison	N/A	N/A
2011	Gatti	Kidney, Ischemic / Reperfusion, Acute Kidney Injury, Chronic Kidney Disease, Nephrectomy	Modified (EVs directly)	EV preconditioning	Enzyme	RNAse	N/A	Apoptosis	Proliferation			Mechanism of action	N/A	N/A	N/A
2012	Reis	Kidney, Acute Kidney Injury	Modified (EVs directly)	EV preconditioning	Enzyme	RNAse	N/A	Necrosis	Proliferation	Apoptosis		Mechanism of action	N/A	N/A	N/A
2012	Zhang	Ischemia (e.g. testing angiogenesis)	Modified (MSCs before EV isolation)	MSC preconditioning	Hypoxia	1% O2	N/A	EV secretion	N/A	N/A		Enhancement	No comparison	N/A	N/A
2012	Bruno	Kidney, Acute Kidney Injury, Nephrotoxicity	Modified (EVs directly)	EV preconditioning	Enzyme	RNAse	N/A	Apoptosis	Proliferation			Mechanism of action	N/A	N/A	N/A
2013	Kilpinen	Kidney, Ischemic / Reperfusion, Acute Kidney Injury	Modified (MSCs before EV isolation)	MSC preconditioning	Inflammatory cytokine	IFN- γ	N/A	Inflammation				Enhancement	Modified < unmodified for most <i>In Vivo</i> outcomes	Worse	N/A
2013	Katakowski	Cancer	Modified (MSCs before EV isolation)	MSC transfection	miRNA	miR-146b	Overexpression	Tumorigenesis				Enhancement	All <i>In Vivo</i> outcomes	Yes	N/A
2013	Roccaro	Cancer	Modified (MSCs before EV isolation), Modified (EVs directly)	MSCs derived from cancer patients + Co-treatment	Cancer + Cells	Multiple myeloma cells co-treated, MSCs from MM patients	N/A	Tumorigenesis				Mechanism of action	N/A	N/A	N/A
2014a	Raisi	Nerve	Modified (MSCs before EV isolation), Modified (EVs directly)	MSC preconditioning + EV Preconditioning	Scaffold (conduit) + Endotoxin	LPS (pro-inflammatory)	N/A	N/A				Enhancement	Modified < unmodified for most <i>In Vivo</i> outcomes	Worse	N/A
2014b	Raisi	Nerve	Modified (MSCs before EV isolation), Modified (EVs directly)	MSC preconditioning + EV Preconditioning	Scaffold (conduit) + Inflammatory Cytokine	PolyI:C (anti-inflammatory)	N/A	Inflammation	Regeneration			Enhancement	Some <i>In Vivo</i> outcomes	No	N/A
2014a	Feng	Heart, Myocardial Infarction (permanent ligation)	Modified (MSCs before EV isolation)	MSC preconditioning	Hypoxia	Anoxia/reoxygenation cycles	N/A	Cell survival	Fibrosis			Enhancement	All <i>In Vivo</i> outcomes	N/A	N/A
2014b	Feng	Heart, Myocardial Infarction (permanent ligation)	Modified (MSCs before EV isolation)	MSC preconditioning + MSC transfection	Hypoxia + miRNA	Anoxia/reoxygenation cycles + miR-22 inhibitor	Knockdown	Cell survival	Fibrosis			Mechanism of action	N/A	N/A	N/A
2014a	Zhu	Lung, Acute Lung Injury	Modified (MSCs before EV isolation)	MSC transfection	protein	KGF siRNA	Knockdown	Inflammation				Mechanism of action	N/A	N/A	N/A

2014b	Zhu	Lung, Acute Lung Injury	Modified (MSCs before EV isolation)	Co-treatment	Protein	anti-KGF neutralizing antibody	Inhibitor / Neutralizing AB	Inflammation				Mechanism of action	N/A	N/A	N/A
2014	Du	Cancer	Modified (EVs directly)	EV preconditioning	Enzyme	RNase	N/A	Tumorigenesis				Mechanism of action	N/A	N/A	N/A
2014	Choi	Kidney, Ischemic / Reperfusion, Acute Kidney Injury	Modified (EVs directly)	EV preconditioning	Enzyme	RNase	N/A	Angiogenesis	apoptosis	Proliferation		Mechanism of action	N/A	N/A	N/A
2014	Bian	Heart, Myocardial Infarction (permanent ligation)	Modified (MSCs before EV isolation)	MSC preconditioning	Hypoxia	1% O2	N/A	N/A	N/A	N/A		N/A	No comparison	N/A	N/A
2015a	Lou	Cancer	Co-injected (e.g. EV + MSCs into animals), Modified (MSCs before EV isolation)	MSC transfection + co-treatment	miRNA + chemotherapeutic	hsa-miR-122 + sorafenib	Overexpression	Chemosensitivity	Tumorigenesis			Enhancement	All <i>In Vivo</i> outcomes	Yes	N/A
2015b	Lou	Cancer	Co-injected (e.g. EV + MSCs into animals), Modified (MSCs before EV isolation)	MSC transfection	miRNA	has-miR-122	Overexpression	Tumorigenesis				Enhancement	No difference	No	N/A
2015a	Monsel	Lung, Acute Lung Injury	Modified (MSCs before EV isolation)	Co-treatment	Protein	KGF neutralizing antibody	Inhibitor / Neutralizing AB	No specific mechanism targeted				Mechanism of action	N/A	N/A	N/A
2015b	Monsel	Lung, Acute Lung Injury	Modified (MSCs before EV isolation)	EV preconditioning	Protein	CD44 neutralizing antibody	Inhibitor / Neutralizing AB	EV incorporation into target cells				Mechanism of action	N/A	N/A	N/A
2015c	Monsel	Lung, Acute Lung Injury	Modified (MSCs before EV isolation)	MSC preconditioning	Inflammatory cytokine	Poly(I:C) (TLR3 agonist)	N/A	Inflammation	Phagocytosis			Enhancement	Some <i>In Vivo</i> outcomes	N/A	N/A
2015a	Zhang	Wound, Angiogenesis (e.g. matrigel plug)	Modified (MSCs before EV isolation)	Co-treatment	Inhibitor	ICG-001 (β -catenin inhibitor)	Inhibitor / Neutralizing AB	Angiogenesis				Mechanism of action	N/A	N/A	N/A
2015b	Zhang	Wound, Angiogenesis (e.g. matrigel plug)	Modified (MSCs before EV isolation)	MSC transduction	Protein	Wnt4 shRNA	Knockdown	Angiogenesis				Mechanism of action	N/A	N/A	N/A
2015a	Zhang	Wound	Co-injected (e.g. EV + MSCs into animals), Modified (MSCs before EV isolation)	Co-treatment	Inhibitor	ICG001 (β -catenin inhibitor)	Inhibitor / Neutralizing AB	Proliferation	Cell migration			Mechanism of action	N/A	N/A	N/A
2015b	Zhang	Wound	Co-injected (e.g. EV + MSCs into animals), Modified (MSCs before EV isolation)	MSC transduction	Protein	Wnt4 shRNA	Knockdown	Proliferation	Cell migration			Mechanism of action	N/A	N/A	N/A
2015c	Zhang	Wound	Co-injected (e.g. EV + MSCs into animals), Modified (MSCs before EV isolation)	Co-treatment	Inhibitor	LY294002 (Akt inhibitor)	Inhibitor / Neutralizing AB	Apoptosis	Proliferation	Cell migration		Mechanism of action	N/A	N/A	N/A
2015	Kang	Heart, Myocardial Infarction (permanent ligation)	Co-injected (e.g. EV + MSCs into animals), Modified (MSCs before EV isolation)	MSC Transfection + Co-treatment	Protein + Cells	CXCR4 + MSC Sheet	Overexpression	Angiogenesis	Apoptosis			Enhancement	All <i>In Vivo</i> outcomes	Yes	N/A
2015	Li	Lung, Acute Lung Injury	Modified (MSCs before EV isolation)	MSC preconditioning	Hypoxia	%O2 not specified	N/A	Inflammation				Enhancement	Most <i>In Vivo</i> outcomes	N/A	N/A
2015	Yang	Ischemic Stroke	Modified (MSCs before EV isolation)	MSC preconditioning	Herbs	BYHWD (Buyang Huanwu decoction)	N/A	Angiogenesis				Enhancement	Unclear	N/A	N/A
2015	Ti	Diabetes, Wound	Modified (MSCs before EV isolation)	MSC preconditioning	Endotoxin	LPS	N/A	Inflammation				Enhancement	Unclear	N/A	N/A
2015	Wang	Sepsis	Modified (MSCs before EV isolation)	MSCs derived from KO animal model	miRNA	miR-223	Knockout	Inflammation				Mechanism of action	N/A	N/A	N/A

2015	Wang	Kidney, Chronic Kidney Disease, Unilateral Ureteral Obstruction (UUO)	Modified (MSCs before EV isolation)	MSC preconditioning	Hormone	EPO	N/A	Fibrosis	Apoptosis		Enhancement	Some <i>In Vivo</i> outcomes	No	N/A	
2015	Choi	Kidney, Chronic Kidney Disease, Unilateral Ureteral Obstruction (UUO)	Modified (EVs directly)	EV preconditioning	Enzyme	RNase	N/A	Fibrosis	Apoptosis	Proliferation	Mechanism of action	N/A	N/A	N/A	
2015	Ju	Kidney, Acute Kidney Injury	Modified (EVs directly)	EV preconditioning	Enzyme	RNase	N/A	Fibrosis	Apoptosis	Proliferation	Mechanism of action	N/A	N/A	N/A	
2015	Yu	Heart, Myocardial Infarction (permanent ligation)	Modified (MSCs before EV isolation)	MSC transduction	Protein	GATA-4	Overexpression	Apoptosis	Fibrosis		Enhancement	Most <i>In Vivo</i> outcomes	Yes	N/A	
2015	Collino	Kidney, Acute Kidney Injury	Modified (MSCs before EV isolation)	MSC transduction	Protein	Drosha shRNA	Knockdown	miRNA depletion	Necrosis		Mechanism of action	N/A	N/A	N/A	
2016a	Wen	Organ transplantation (e.g. solid organs, skin grafts, etc.), Diabetes	Modified (MSCs before EV isolation)	MSC preconditioning	Co-culture of cells	PBMCs	N/A	Inflammation			Enhancement	No Difference	N/A	N/A	
2016b	Wen	Organ transplantation (e.g. solid organs, skin grafts, etc.), Diabetes	Modified (MSCs before EV isolation)	Co-treatment + MSC preconditioning	cells + Co-culture of cells	Co-treatment: transplantation of MSCs transfected with anti-miR-375 and pshFas; MSC preconditioning : PBMCs	N/A	Inflammation	Apoptosis	Insulin dependence	Enhancement	No comparison	N/A	N/A	
2016a	Vrijssen	Angiogenesis (e.g. matrigel plug)	Co-injected (e.g. EV + MSCs into animals)	EV preconditioning	Protein	αEMMPRIN neutralizing antibody	Inhibitor / Neutralizing AB	Angiogenesis			Mechanism of action	N/A	N/A	N/A	
2016b	Vrijssen	Angiogenesis (e.g. matrigel plug)	Co-injected (e.g. EV + MSCs into animals)	MSC transduction	Protein	EMMPRIN shRNA	Knockdown	Angiogenesis			Mechanism of action	N/A	N/A	N/A	
2016	Yu	Eye	Co-injected (e.g. EV + MSCs into animals)	Co-treatment	Inflammatory cytokine	MCP-1	N/A	Inflammation			Mechanism of action	N/A	N/A	N/A	
2016	Shen	Kidney, Ischemic / Reperfusion	Modified (MSCs before EV isolation)	MSC transfection	Protein	CCR-2 siRNA	Knockdown	Inflammation			Mechanism of action	N/A	N/A	N/A	
2016	Zou	Kidney, Ischemic / Reperfusion	Modified (EVs directly)	EV preconditioning	Enzyme	RNase	N/A	Apoptosis	Angiogenesis	Fibrosis	Mechanism of action	N/A	N/A	N/A	
2016	Gu	Kidney, Ischemic / Reperfusion	Modified (MSCs before EV isolation)	MSC transfection	miRNA	miR-30b, miR-30c, and/or miR-30d antagonists	Knockdown	Mitochondrial function	Apoptosis		Mechanism of action	N/A	N/A	N/A	
2016	Lee	Ischemic Stroke	Modified (MSCs before EV isolation)	MSC preconditioning	Brain extract	Normal rat brain extract or stroke-injured rat brain extract	N/A	Inflammation	Neurogenesis		Enhancement	Some <i>In Vivo</i> outcomes	No	N/A	
2016	Xie	Bone/Joint	Co-injected (e.g. EV + MSCs into animals), Modified (EVs directly)	EV Preconditioning	Scaffold + Alginate	Alginate solution and polycaprolactone scaffold	N/A	N/A	N/A	N/A	N/A	No comparison	N/A	N/A	
2016	Kalimuthu	Cancer	Modified (MSCs before EV isolation)	MSC transduction	Protein	Renilla luciferase	Overexpression	N/A			Visualization	No comparison	N/A	N/A	
2016	Chen	Ischemic Stroke	Co-injected (e.g. EV + MSCs into animals)	Co-treatment	Cells	Adipose-derived MSCs	N/A	Angiogenesis	Inflammation	Fibrosis	Mitochondrial Function	Enhancement	Most <i>In Vivo</i> outcomes	No	N/A
2016	Liu	Aneurysm	Co-injected (e.g. EV + MSCs into animals)	Co-treatment	Inhibitor	GW 627368X (EP4 receptor antagonist)	Inhibitor / Neutralizing AB	Inflammation			Mechanism of action	N/A	N/A	N/A	

2016	Zhang	Wound	Modified (MSCs before EV isolation)	MSC transduction	Protein	14-3-3 ζ shRNA	Knockdown	Stem cell expansion	Collagen deposition		Mechanism of action	N/A	N/A	N/A	
2016	Li	Wound	Modified (MSCs before EV isolation)	MSC transfection	miRNA	miR-181c	Overexpression	Inflammation			Enhancement	Most <i>In Vivo</i> outcomes	N/A	N/A	
2016	Fang	Wound	Modified (EVs directly)	EV transfection	miRNA	miR-21, miR-23a, miR-125b, and miR-145 antagomirs	Knockdown	Myofibroblast formation			Mechanism of action	N/A	N/A	N/A	
2016	Qi	Bone/Joint	Modified (EVs directly)	EV Preconditioning	Scaffold	β -TCP scaffold	N/A	N/A	N/A	N/A	N/A	No comparison	N/A	N/A	
2016	Qin	Bone/Joint	Modified (EVs directly)	EV Preconditioning	Scaffold	HyStem-HP hydrogel	N/A	N/A	N/A	N/A	N/A	No comparison	N/A	N/A	
2016	Ti	Diabetes, Wound	Modified (MSCs before EV isolation)	MSC preconditioning	Endotoxin	LPS	N/A	Inflammation	Regeneration		Enhancement	Unclear	N/A	N/A	
2017a	Gonzalez-King	Angiogenesis (e.g. matrigel plug)	Modified (MSCs before EV isolation)	MSC transduction	Protein	HIF-1 α	Overexpression	Angiogenesis			Enhancement	All <i>In Vivo</i> outcomes	N/A	N/A	
2017b	Gonzalez-King	Angiogenesis (e.g. matrigel plug)	Modified (MSCs before EV isolation)	MSC transduction + EV preconditioning	Protein + Protein	HIF-1 α + Jagged1 neutralizing antibody	Overexpression + Inhibitor Neutralizing AB	Angiogenesis			Mechanism of action	N/A	N/A	N/A	
2017c	Gonzalez-King	Angiogenesis (e.g. matrigel plug)	Modified (MSCs before EV isolation)	EV preconditioning	Protein	Jagged1 neutralizing antibody	Inhibitor / Neutralizing AB	Angiogenesis			Mechanism of action	N/A	N/A	N/A	
2017a	Song	Sepsis	Modified (MSCs before EV isolation)	MSC preconditioning	Inflammatory cytokine	IL-1 β	N/A	Inflammation			Enhancement	All <i>In Vivo</i> outcomes	N/A	Yes	
2017b	Song	Sepsis	Modified (MSCs before EV isolation)	MSC transfection	Inflammatory cytokine + miRNA	miR-146a mimic	Overexpression	Inflammation			Enhancement	Most <i>In Vivo</i> outcomes	N/A	Yes	
2017c	Song	Sepsis	Modified (MSCs before EV isolation)	MSC preconditioning + MSC transfection	Inflammatory cytokine + miRNA	IL-1 β + miR-146a inhibitor	Knockdown	Inflammation			Mechanism of action	N/A	N/A	N/A	
2017a	Kamerkar	Cancer	Co-injected (e.g. EV + MSCs into animals), Modified (EVs directly)	MSC transfection	protein	KRAS ^{G12D} shRNA	Overexpression	Tumorigenesis			Enhancement	Most <i>In Vivo</i> outcomes	N/A	Yes	
2017b	Kamerkar	Cancer	Co-injected (e.g. EV + MSCs into animals), Modified (EVs directly)	MSC transfection + EV preconditioning	protein + enzyme	KRAS ^{G12D} shRNA + Proteinase K + RNase A	Overexpression + Knockdown	Tumorigenesis			Mechanism of action	N/A	N/A	N/A	
2017c	Kamerkar	Cancer	Co-injected (e.g. EV + MSCs into animals), Modified (EVs directly)	MSC transfection + EV preconditioning	protein + enzyme	KRAS ^{G12D} shRNA + RNase A	Overexpression + Knockdown	Tumorigenesis			Mechanism of action	N/A	N/A	N/A	
2017d	Kamerkar	Cancer	Co-injected (e.g. EV + MSCs into animals), Modified (EVs directly)	MSC transfection + EV preconditioning	protein + protein	KRAS ^{G12D} shRNA + CD47 neutralizing antibody	Overexpression + inhibitor / neutralizing AB	Tumorigenesis	Phagocytosis		Mechanism of action	N/A	N/A	N/A	
2017e	Kamerkar	Cancer	Co-injected (e.g. EV + MSCs into animals), Modified (EVs directly)	MSC transfection + MSCs derived from KO animal model	protein + protein	KRAS ^{G12D} shRNA + CD47	Overexpression	Tumorigenesis	Phagocytosis		Mechanism of action	N/A	N/A	N/A	
2017a	Du	Liver	Co-injected (e.g. EV + MSCs into animals)	Co-treatment	Inhibitor	SKII (SK inhibitor)	Inhibitor / Neutralizing AB	Proliferation			Mechanism of action	N/A	N/A	N/A	
2017b	Du	Liver	Co-injected (e.g. EV + MSCs into animals)	Co-treatment	Inhibitor	VPC23019 (S1P1 receptor antagonist)	Inhibitor / Neutralizing AB	Proliferation			Mechanism of action	N/A	N/A	N/A	
2017a	de Almeida	Kidney, Acute Kidney Injury, Nephrotoxicity	Modified (EVs directly)	EV preconditioning	Enzyme	RNase A + Proteinase K	N/A	Apoptosis	Proliferation		Mechanism of action	N/A	N/A	N/A	
2017b	de Almeida	Kidney, Acute Kidney Injury, Nephrotoxicity	Modified (EVs directly)	EV preconditioning	Enzyme	DNase I + Proteinase K	N/A	Apoptosis	Proliferation		Mechanism of action	N/A	N/A	N/A	
2017c	de Almeida	Kidney, Acute Kidney Injury, Nephrotoxicity	Modified (EVs directly)	EV preconditioning	Enzyme	Proteinase K + trypsin + collagenase 1A	N/A	Apoptosis	Proliferation		Mechanism of action	N/A	N/A	N/A	
2017	Cheng	Intervertebral disc degeneration	Modified (MSCs before EV isolation)	MSC transfection	miRNA	agomiR-21	Overexpression	Cell survival	Apoptosis		Mechanism of action	N/A	N/A	N/A	
2017	Zhu	Heart, Myocardial Infarction	Modified (MSCs before EV isolation)	MSC preconditioning	Hypoxia	0.5% O2	N/A	EV secretion	Angiogenesis	Apoptosis	Cell migration	Enhancement	Most <i>In Vivo</i> outcomes	Yes	Yes

		(permanent ligation)													
2017	Mead	Eye	Modified (MSCs before EV isolation)	MSC transfection	Protein/miRNA (Ago2)	Argonaute 2 siRNA	Knockdown	Neuroprotection	Regeneration			Mechanism of action	N/A	N/A	N/A
2017	Yuan	Kidney, Ischemic / Reperfusion	Modified (MSCs before EV isolation)	KO iPSC line	Protein	SP1	Knockout	Necroptosis				Mechanism of action	N/A	N/A	N/A
2017	Chen	Kidney, Ischemic / Reperfusion	Co-injected (e.g. EV + MSCs into animals)	Co-treatment	Inhibitor	U0126	Inhibitor / Neutralizng AB	Cell cycle progression	Fibrosis	Proliferation	Apoptosis	Mechanism of action	N/A	N/A	N/A
2017	Zhang	Traumatic Brain Injury	Modified (MSCs before EV isolation)	MSC preconditioning	Scaffold	Ultrafoam scaffold	N/A	3D culture	Angiogenesis	Neurogenesis	Inflammation	Enhancement	Some <i>In Vivo</i> outcomes	No	N/A
2017	Shi	Diabetes, Wound	Modified (EVs directly)	EV Preconditioning	Scaffold	Chitosan/silk hydrogel sponge	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A
2017	Gangadaran	Ischemia (e.g. testing angiogenesis)	Modified (EVs directly)	EV Preconditioning	Scaffold	Matrigel	N/A	Angiogenesis	Retention			Enhancement	Most <i>In Vivo</i> outcomes	Yes	N/A
2017	Sun	Liver	Co-injected (e.g. EV + MSCs into animals)	Co-treatment	Hormone	Melatonin	N/A	Oxidative stress	Inflammation	Apoptosis	Mitochondrial Function	Enhancement	All <i>In Vivo</i> outcomes	Yes	N/A
2017	Qu	Liver	Modified (MSCs before EV isolation)	MSC transfection	miRNA	miR-181-5p	Overexpression	Fibrosis	Autophagy	Inflammation		Enhancement	Some <i>In Vivo</i> outcomes	Yes	N/A
2017	Xie	Bone/Joint	Modified (EVs directly)	EV Preconditioning	Scaffold	Decalcified bone matrix	N/A	N/A	N/A	N/A		N/A	N/A	N/A	N/A
2017	Wang	Heart, Myocardial Infarction (permanent ligation)	Modified (MSCs before EV isolation)	MSC transfection	miRNA	miR-210 siRNA	Knockdown	Angiogenesis	Fibrosis			Mechanism of action	N/A	N/A	N/A
2017	Ma	Heart, Myocardial Infarction (permanent ligation)	Modified (MSCs before EV isolation)	MSC transduction	Protein	Akt	Overexpression	Angiogenesis	Cell migration			Enhancement	Most <i>In Vivo</i> outcomes	Yes	N/A
2017	Li	Bone/Joint	Modified (MSCs before EV isolation)	MSC transduction	Protein (mutation)	Mutant HIF-1α	Mutation	Osteogenesis	Angiogenesis			Enhancement	Most <i>In Vivo</i> outcomes	N/A	N/A
2017	Eirin	Kidney, Chronic Kidney Disease	Modified (MSCs before EV isolation)	MSC transfection	Protein	IL-10 siRNA	Knockdown	Inflammation				Mechanism of action	N/A	N/A	N/A
2017	Tang	Lung, Acute Lung Injury	Modified (MSCs before EV isolation)	MSC transduction	Protein	Ang-1 shRNA	Knockdown	Vascular stabilization	Inflammation			Mechanism of action	N/A	N/A	N/A
2017	Du	Ischemia (e.g. testing angiogenesis)	Modified (MSCs before EV isolation)	MSC preconditioning	Polymer	Chitosan NO-releasing polymer	N/A	Angiogenesis				Enhancement	All <i>In Vivo</i> outcomes	Yes	N/A
2017	Moisseiev	Eye	Modified (MSCs before EV isolation)	MSC preconditioning	Hypoxia	1% O2	N/A	N/A	N/A	N/A		N/A	No comparison	N/A	N/A
2017	Liu	Heart, Myocardial infarction (ischemia-reperfusion)	Modified (MSCs before EV isolation)	MSC preconditioning	Hypoxia	1% O2	N/A	N/A	N/A	N/A		N/A	No comparison	N/A	N/A
2017	Xin	Ischemic Stroke	Modified (MSCs before EV isolation)	MSC transduction	miRNA	pre-miR-133b	Overexpression	Neurite remodeling	Retention			Enhancement	All <i>In Vivo</i> outcomes	Yes	N/A
2017	Yan	Liver	Modified (MSCs before EV isolation)	MSC transfection	Protein	GPX1 siRNA	Knockdown	Oxidative stress	Apoptosis			Mechanism of action	N/A	N/A	N/A
2017	Tao	Bone/Joint	Modified (MSCs before EV isolation)	MSC transduction	miRNA	miR-140-5p	Overexpression	Proliferation	Cell migration	Collagen Deposition		Enhancement	All <i>In Vivo</i> outcomes	N/A	N/A
2017	Tao	Diabetes, Wound	Modified (MSCs before EV isolation), Modified (EVs directly)	EV Preconditioning + MSC transduction	Scaffold + miRNA	Chitosan gel + miR-126-3p	Overexpression	Angiogenesis	Regeneration			Enhancement	No comparison	N/A	N/A
2017	Tamura	Liver	Modified (EVs directly)	EV preconditioning	Polysaccharide	Cationized pullulan	N/A	EV incorporation into target cells	Inflammation	Necrosis		Enhancement	Most <i>In Vivo</i> outcomes	Yes	N/A
2017	Ranghino	Kidney, Ischemic /	Modified (EVs directly)	EV preconditioning	Enzyme	RNase	N/A	Necrosis	Proliferation	Apoptosis		Mechanism of action	N/A	N/A	N/A

		Reperfusion, Acute Kidney Injury, Nephrectomy												
2017	Xin	Ischemic Stroke	Modified (MSCs before EV isolation)	MSC transfection	miRNA	miR-17-92 cluster	Overexpression	Neurogenesis	Neurite remodeling		Enhancement	All <i>In Vivo</i> outcomes	Yes	N/A
2017	Lo Sicco	Muscle, Angiogenesis (e.g. matrigel plug)	Modified (MSCs before EV isolation)	MSC preconditioning	Hypoxia	%O2 not specified	N/A	Inflammation	Angiogenesis	Myogenesis		Enhancement	Unclear	N/A
2018	Luther	Heart, Myocardial infarction (ischemia-reperfusion)	Modified (MSCs before EV isolation)	MSCs derived from KO animal model	miRNA	miR-21a	Knockout	Apoptosis				Mechanism of action	N/A	N/A
2018	Ahn	Lung, BPD	Modified (MSCs before EV isolation)	MSC transfection	Protein	VEGF siRNA	Knockdown	Angiogenesis	Apoptosis	Inflammation		Mechanism of action	N/A	N/A
2018	Blazquez	Incisional hernia	Modified (EVs directly)	EV Preconditioning	Scaffold	Fibring glue + surgical mesh	N/A	N/A				N/A	N/A	N/A
2018	Shen	Hemorrhagic Stroke (e.g. Intracranial Hemorrhage)	Modified (MSCs before EV isolation)	MSC transfection	miRNA	miR-133b	Overexpression	Apoptosis				Enhancement	All <i>In Vivo</i> outcomes	N/A
2018	Lang	Cancer	Modified (MSCs before EV isolation)	MSC transduction	miRNA	miR-124a	Overexpression	Tumorigenesis	Lipid metabolism			Enhancement	All <i>In Vivo</i> outcomes	Yes
2018	Cui	Cognitive impairment (e.g. Alzheimer's, Parkinson's Disease, etc.)	Modified (MSCs before EV isolation)	MSC preconditioning	Hypoxia	0% O2	N/A	N/A	apoptosis	inflammation	plaque deposition	Enhancement	Most <i>In Vivo</i> outcomes	Yes
2018	Li	Bone/Joint	Modified (EVs directly)	EV Preconditioning	Scaffold	Poly(lactic-co-glycolic acid) (PLGA) or polydopamine-coated PLGA (pDA/PLGA)	N/A	N/A				N/A	N/A	N/A
2018	Mead	Eye	Modified (MSCs before EV isolation)	MSC transfection	Protein/miRNA (Ago2)	Argonaute 2 siRNA	Knockdown	miRNA depletion				Mechanism of action	N/A	N/A
2018	Ruppert	Spinal cord injury	Modified (MSCs before EV isolation)	MSC preconditioning	Inflammatory cytokine	TNF- α + IFN- γ	N/A	Inflammation				Enhancement	Some <i>In Vivo</i> outcomes	Yes
2018	Han	Organ transplantation (e.g. solid organs, skin grafts, etc.)	Modified (MSCs before EV isolation)	MSC preconditioning	Hypoxia	5% O2	N/A	Angiogenesis	Cell survival	Inflammation		Enhancement	Most <i>In Vivo</i> outcomes	N/A
2018	O'Brien	Cancer	Modified (MSCs before EV isolation)	MSC transduction	miRNA	miR-379	Overexpression	Tumorigenesis				Enhancement	All <i>In Vivo</i> outcomes	Yes
2018	Chang	Sepsis	Modified (MSCs before EV isolation)	MSC preconditioning	Hypoxia/starvation	1% O2 followed by serum deprivation	N/A	Inflammation	Oxidative stress			Enhancement	Modified < unmodified for most <i>In Vivo</i> outcomes	Worse
2018	Tian	Bone/Joint	Modified (EVs directly), Modified (MSCs before EV isolation)	EV Preconditioning + MSC Transfection	Scaffold + Protein (Mutation)	Scaffold / HIF1a	Mutation	Apoptosis	Inflammation	Regeneration		Enhancement	All <i>In Vivo</i> outcomes	N/A
2018	Chen	Diabetes, Wound	Modified (MSCs before EV isolation)	MSC transduction	Protein	shDMBT1	Knockdown	Angiogenesis	Proliferation	Collagen Deposition		Mechanism of action	N/A	N/A
2018	Jia	Kidney, Acute Kidney Injury, Nephrotoxicity	Modified (MSCs before EV isolation)	MSC transduction	Protein	14-3-3 ζ	Overexpression	Autophagy	Proliferation	Apoptosis		Enhancement	All <i>In Vivo</i> outcomes	N/A
2018a	Diomedea	Bone/Joint	Co-injected (e.g. EV + MSCs into animals), Modified (EVs directly)	EV preconditioning	Polymer	Polyethylencimine	N/A	EV incorporation into target cells	osteogenesis	angiogenesis		Enhancement	Unclear	N/A
2018b	Diomedea	Bone/Joint	Co-injected (e.g. EV + MSCs into animals), Modified (EVs directly)	Co-treatment	Cells	Human gingival MSCs	N/A	N/A				Enhancement	Unclear	N/A

2018a	Chen	Liver	Modified (MSCs before EV isolation)	MSC transduction	miRNA	pre-miR-223	Overexpression	Inflammation	Apoptosis		Enhancement	All <i>In Vivo</i> outcomes	Yes	N/A
2018b	Chen	Liver	Modified (MSCs before EV isolation)	MSC transfection	miRNA	miR-223 inhibitor	Knockdown	Inflammation	Apoptosis		Mechanism of action	N/A	N/A	N/A
2018a	Kou	Diabetes, Wound	Modified (MSCs before EV isolation)	KO animal model + MSCs derived from KO animal model	Anti-inflammatory cytokine	IL-1RA (IL-1 receptor antagonist)	Inhibitor / Neutralizing AB	Inflammation			Mechanism of action	N/A	N/A	N/A
2018b	Kou	Diabetes, Wound	Modified (MSCs before EV isolation)	MSC preconditioning	Inflammatory cytokine	TNF- α	N/A	Inflammation	Regeneration		Enhancement	All <i>In Vivo</i> outcomes	Yes	N/A
2018c	Kou	Diabetes, Wound	Modified (MSCs before EV isolation)	MSCs derived from KO animal model	Anti-inflammatory cytokine	IL-1RA (IL-1 receptor antagonist)	Inhibitor / Neutralizing AB	Inflammation			Mechanism of action	N/A	N/A	N/A

Supplemental File B6 – PRISMA Checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	N/A
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	Page 2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	Page 3-5
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	Pages 5
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g. Web address), and, if available, provide registration information including registration number.	Page 22-23
Eligibility criteria	6	Specify study characteristics (e.g. PICOS, length of follow-up) and report characteristics (e.g. years considered, language, publication status) used as criteria for eligibility, giving rationale.	Page 23-24
Information sources	7	Describe all information sources (e.g. databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	Page 24
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Page 24, Suppl. File 7
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	Pages 24
Data collection process	10	Describe method of data extraction from reports (e.g. piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	Pages 25-27
Data items	11	List and define all variables for which data were sought (e.g. PICOS, funding sources) and any assumptions and simplifications made.	Pages 25-27
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	N/A
Summary measures	13	State the principal summary measures (e.g. risk ratio, difference in means).	Page 26-27
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g. I^2) for each meta-analysis.	Page 26-27

Supplemental File B7 – Search Strategy

Database: Ovid MEDLINE(R) ALL

Search Strategy:

-
- 1 Mesenchymal Stromal Cells/
 - 2 Mesenchymal Stem Cell Transplantation/
 - 3 Multipotent Stem Cells/
 - 4 (MSC or MSCs or ADMSC or ADMSCs or BM-MSC or BM-MSCs or BMD-MSC or BMD-MSCs or BMDMSC or BMDMSCs).tw,kw.
 - 5 (mesenchymal adj5 (cell* or stem or stromal or progenitor* or multipotent or bone marrow or adipose or placenta*)).tw.
 - 6 (mesenchymal and (cell* or stem or stromal or progenitor or multipotent or bone marrow or adipose or placenta*)).kw.
 - 7 ((multipotent or multi-potent) adj (stroma* cell* or stem cell*)).tw.
 - 8 ((multipotent or multi-potent) and (stroma* cell* or stem cell*)).kw.
 - 9 (colony-forming adj2 fibroblast*).tw.
 - 10 marrow stroma* cell*.tw.
 - 11 Mesoderm/cy
 - 12 or/1-11
 - 13 Exosomes/ or Extracellular Vesicles/
 - 14 exosom*.tw,kw.
 - 15 Cell-Derived Microparticles/
 - 16 (microvesicle* or micro vesicle*).tw,kw.
 - 17 ((cell or cells or extracellular) adj2 vesicle*).tw.
 - 18 microparticle*.tw,kw.
 - 19 ((cell or cells or extracellular) and vesicle*).kw.
 - 20 or/13-19
 - 21 12 and 20
 - 22 exp "animal experimentation"/ or exp "models, animal"/ or exp "invertebrates"/ or "Animals"/ or exp "animal population groups"/ or "chordata"/ or exp "chordata, nonvertebrate"/ or "vertebrates"/ or exp "amphibians"/ or exp "birds"/ or exp "fishes"/ or exp "reptiles"/ or "mammals"/ or "primates"/ or exp "artiodactyla"/ or exp "carnivora"/ or exp "cetacea"/ or exp "chiroptera"/ or exp "elephants"/ or exp "hyraxes"/ or exp "insectivora"/ or exp "lagomorpha"/ or exp "marsupialia"/ or exp "monotremata"/ or exp "perissodactyla"/ or exp "rodentia"/ or exp "scandentia"/ or exp "sirenia"/ or exp "xenarthra"/ or "haplorhini"/ or exp "strepsirhini"/ or exp "platyrrhini"/ or exp "tarsii"/ or "catarrhini"/ or exp "cercopithecidae"/ or exp "hylobatidae"/ or "hominidae"/ or exp "gorilla gorilla"/ or exp "pan paniscus"/ or exp "pan troglodytes"/ or exp "pongo pygmaeus"/ (6252795)
 - 23 (animals or animal or mice or mus or mouse or murine or woodmouse or rats or rat or murinae or muridae or cottonrat or cottonrats or hamster or hamsters or cricetinae or rodentia or rodent or rodents or pigs or pig or swine or swines or piglets or piglet or boar or boars or "sus scrofa" or ferrets or ferret or polecat or polecats or "mustela putorius" or "guinea pigs" or "guinea pig" or cavia or callithrix or marmoset or marmosets or

cebuella or hapale or octodon or chinchilla or chinchillas or gerbillinae or gerbil or gerbils or jird or jirds or merione or meriones or rabbits or rabbit or hares or hare or diptera or flies or fly or dipteral or drosophila or drosophilidae or cats or cat or carus or felis or nematoda or nematode or nematoda or nematode or nematodes or sipunculida or dogs or dog or canine or canines or canis or sheep or sheeps or mouflon or mouflons or ovis or goats or goat or capra or capras or rupicapra or chamois or haplorhini or monkey or monkeys or anthropoidea or anthropoids or saguinus or tamarin or tamarins or leontopithecus or hominidae or ape or apes or pan or paniscus or "pan paniscus" or bonobo or bonobos or troglodytes or "pan troglodytes" or gibbon or gibbons or siamang or siamangs or nomascus or symphalangus or chimpanzee or chimpanzees or prosimians or "bush baby" or prosimian or bush babies or galagos or galago or pongidae or gorilla or gorillas or pongo or pygmaeus or "pongo pygmaeus" or orangutans or pygmaeus or lemur or lemurs or lemuridae or horse or horses or pongo or equus or cow or calf or bull or chicken or chickens or gallus or quail or bird or birds or quails or poultry or poultries or fowl or fowls or reptile or reptilia or reptiles or snakes or snake or lizard or lizards or alligator or alligators or crocodile or crocodiles or turtle or turtles or amphibian or amphibians or amphibia or frog or frogs or bombina or salientia or toad or toads or "epidalea calamita" or salamander or salamanders or eel or eels or fish or fishes or pisces or catfish or catfishes or siluriformes or arius or heteropneustes or sheatfish or perch or perches or percidae or perca or trout or trouts or char or chars or salvelinus or "fathead minnow" or minnow or cyprinidae or carps or carp or zebrafish or zebrafishes or goldfish or goldfishes or guppy or guppies or chub or chubs or tinca or barbels or barbus or pimephales or promelas or "poecilia reticulata" or mullet or mullets or seahorse or seahorses or mugil curema or atlantic cod or shark or sharks or catshark or anguilla or salmonid or salmonids or whitefish or whitefishes or salmon or salmons or sole or solea or "sea lamprey" or lamprey or lampreys or pumpkinseed or sunfish or sunfishes or tilapia or tilapias or turbot or turbots or flatfish or flatfishes or sciuridae or squirrel or squirrels or chipmunk or chipmunks or suslik or susliks or vole or voles or lemming or lemmings or muskrat or muskrats or lemmus or otter or otters or marten or martens or martes or weasel or badger or badgers or ermine or mink or minks or sable or sables or gulo or gulos or wolverine or wolverines or minks or mustela or llama or llamas or alpaca or alpacas or camelid or camelids or guanaco or guanacos or chiroptera or chiropteras or bat or bats or fox or foxes or iguana or iguanas or xenopus laevis or parakeet or parakeets or parrot or parrots or donkey or donkeys or mule or mules or zebra or zebras or shrew or shrews or bison or bisons or buffalo or buffaloes or deer or deers or bear or bears or panda or pandas or "wild hog" or "wild boar" or fitchew or fitch or beaver or beavers or jerboa or jerboas or capybara or capybaras).tw.

24 Drug Evaluation, Preclinical/

25 (preclinic* or pre clinic*).tw,kw.

26 22 or 23 or 24 or 25

27 21 and 26

Database: Embase Classic+Embase

Search Strategy:

1 exp mesenchymal stem cell/

2 exp mesenchymal stem cell transplantation/
 3 exp mesenchymal stroma cell/
 4 (MSC or MSCs or ADMSC or ADMSCs or BM-MSC or BM-MSCs or BMD-MSC or BMD-MSCs or BMDMSC or BMDMSCs or BMSC or
 BMSCs or HBMSC or HBMSCs or ABMSC or ABMSCs or MAPC or MAPCs).tw.
 5 (((multipotent or multi-potent) adj3 (stem or stroma\$1 or progenitor*)) and (cell\$1 or cell-induced)).tw.
 6 multipotent stem cell/
 7 (mesenchymal adj5 (cell* or stem or stroma* or progenitor* or multipotent or bone marrow or adipose or placenta*)).tw.
 8 (colony-forming adj2 fibroblast*).tw.
 9 (marrow stroma* adj2 cell*).tw.
 10 or/1-9
 11 exosome/
 12 exosom*.tw.
 13 (microvesicle* or micro vesicle*).tw.
 14 microparticle*.tw.
 15 ((cell or cells or extracellular) adj2 vesicle*).tw.
 16 exp membrane microparticle/
 17 or/11-16
 18 10 and 17
 19 exp animal experiment/ or exp animal model/ or exp experimental animal/ or exp transgenic animal/ or exp male animal/ or exp female animal/
 or exp juvenile animal/ or animal/ or chordata/ or vertebrate/
 20 tetrapod/ or exp fish/ or amniote/ or exp amphibia/ or mammal/ or exp reptile/ or exp sauropsid/ or therian/ or exp monotremate/ or placental
 mammals/ or exp marsupial/ or Euarchontoglires/ or exp Afrotheria/ or exp Boreoeutheria/ or exp Laurasiatheria/ or exp Xenarthra/ or primate/ or
 exp Dermoptera/ or exp Glires/ or exp Scandentia/ or Haplorhini/ or exp prosimian/ or simian/ or exp tarsiiiform/ or Catarrhini/ or exp Platyrrhini/
 or ape/ or exp Cercopithecidae/ or hominid/ or exp hylobatidae/ or exp chimpanzee/ or exp gorilla/ or exp orang utan/
 21 (preclinic* or pre clinic*).tw.
 22 (animal or animals or pisces or fish or fishes or catfish or catfishes or sheatfish or silurus or arius or heteropneustes or clarias or gariepinus or
 fathead minnow or fathead minnows or pimephales or promelas or cichlidae or trout or trouts or char or chars or salvelinus or salmo or oncorhynchus
 or guppy or guppies or millionfish or poecilia or goldfish or goldfishes or carassius or auratus or mullet or mullets or mugil or curema or shark or
 sharks or cod or cods or gadus or morhua or carp or carps or cyprinus or carpio or killifish or eel or eels or anguilla or zander or sander or lucioperca
 or stizostedion or turbot or turbot or psetta or flatfish or flatfishes or plaice or pleuronectes or platessa or tilapia or tilapias).tw.
 23 (oreochromis or sarotherodon or common sole or dover sole or solea or zebrafish or zebrafishes or danio or rerio or seabass or dicentrarchus
 or labrax or morone or lamprey or lampreys or petromyzon or pumpkinseed or pumpkinseeds or lepomis or gibbosus or herring or clupea or harengus
 or amphibia or amphibian or amphibians or anura or salientia or frog or frogs or rana or toad or toads or bufo or xenopus or laevis or bombina or
 epidalea or calamita or salamander or salamanders or newt or newts or triturus or reptilia or reptile or reptiles or bearded dragon or pogona or
 vitticeps or iguana or iguanas or lizard).tw.

24 (lizards or anguis fragilis or turtle or turtles or snakes or snake or aves or bird or birds or quail or quails or coturnix or bobwhite or colinus or virginianus or poultry or poultries or fowl or fowls or chicken or chickens or gallus or zebra finch or taeniopygia or guttata or canary or canaries or serinus or canaria or parakeet or parakeets or grasskeet or parrot or parrots or psittacine or psittacines or shelduck or tadorna or goose or geese or branta or leucopsis or woodlark or lullula or flycatcher or ficedula or hypoleuca or dove or doves or geopelia or cuneata or duck or ducks or greylag or graylag or anser or harrier or circus pygargus or red knot or great knot or calidris or canutus or godwit or limosa or lapponica or meleagris or gallopavo or jackdaw or corvus or monedula or ruff or philomachus or pugnax or lapwing or peewit or plover or vanellus or swan or cygnus or columbianus or bewickii or gull or chroicocephalus or ridibundus).tw.

25 (albifrons or great tit or parus or aythya or fuligula or streptopelia or risoria or spoonbill or platalea or leucorodia or blackbird or turdus or merula or blue tit or cyanistes or pigeon or pigeons or columba or pintail or anas or starling or sturnus or owl or athene noctua or pochard or ferina or cockatiel or nymphicus or hollandicus or skylark or alauda or tern or sterna or teal or crecca or oystercatcher or haematopus or ostralegus or shrew or shrews or sorex or araneus or crocidura or russula or european mole or talpa or chiroptera or bat or bats or eptesicus or serotinus or myotis or dasycneme or daubentonii or pipistrelle or pipistrellus or cat or cats or felis or catus or feline or dog or dogs or canis or canine or canines or otter or otters or lutra or badger or badgers or meles or fitchew or fitch or foudmart or foulmart or ferrets or ferret or polecat or polecats or mustela or putorius or weasel or weasels or fox or foxes or vulpes or common seal or phoca or vitulina or grey seal or halichoerus or horse or horses or equus or equine or equidae or donkey or donkeys or mule or mules or pig or pigs or swine or swines or hog or hogs or boar or boars or porcine or piglet or piglets or sus or scrofa or llama or llamas or lama or glama or deer or deers or cervus or elaphus or cow or cows or bos taurus or bos indicus or bovine or bull or bulls).tw.

26 (cattle or bison or bisons or sheep or sheeps or ovis aries or ovine or lamb or lambs or mouflon or mouflons or goat or goats or capra or caprine or chamois or rupicapra or leporidae or lagomorpha or lagomorph or rabbit or rabbits or oryctolagus or cuniculus or laprine or hares or lepus or rodentia or rodent or rodents or murinae or mouse or mice or mus or musculus or murine or woodmouse or apodemus or rat or rats or rattus or norvegicus or guinea pig or guinea pigs or cavia or porcellus or hamster or hamsters or mesocricetus or cricetus or cricetus or gerbil or gerbils or jird or jirds or meriones or unguiculatus or jerboa or jerboas or jaculus or chinchilla or chinchillas or beaver or beavers or castor fiber or castor canadensis or sciuridae or squirrel or squirrels or sciurus or chipmunk or chipmunks or marmot or marmots or marmota or suslik or susliks or spermophilus or cynomys or cottonrat or cottonrats or sigmodon or vole or voles or microtus or myodes or glareolus or primate).tw.

27 (primates or prosimian or prosimians or lemur or lemurs or lemuridae or loris or bush baby or bush babies or bushbaby or bushbabies or galago or galagos or anthropoidea or anthropoids or simian or simians or monkey or monkeys or marmoset or marmosets or callithrix or cebuella or tamarin or tamarins or saguinus or leontopithecus or squirrel monkey or squirrel monkeys or saimiri or night monkey or night monkeys or owl monkey or owl monkeys or douroucoulis or aotus or spider monkey or spider monkeys or ateles or baboon or baboons or papio or rhesus monkey or macaque or macaca or mulatta or cynomolgus or fascicularis or green monkey or green monkeys or chlorocebus or vervet or vervets or pygerythrus or hominoidea or ape or apes or hylobatidae or gibbon or gibbons or siamang or siamangs or nomascus or symphalangus or hominidae or orangutan or orangutans or pongo or chimpanzee or chimpanzees or pan troglodytes or bonobo or bonobos or pan paniscus or gorilla or gorillas or troglodytes).tw.

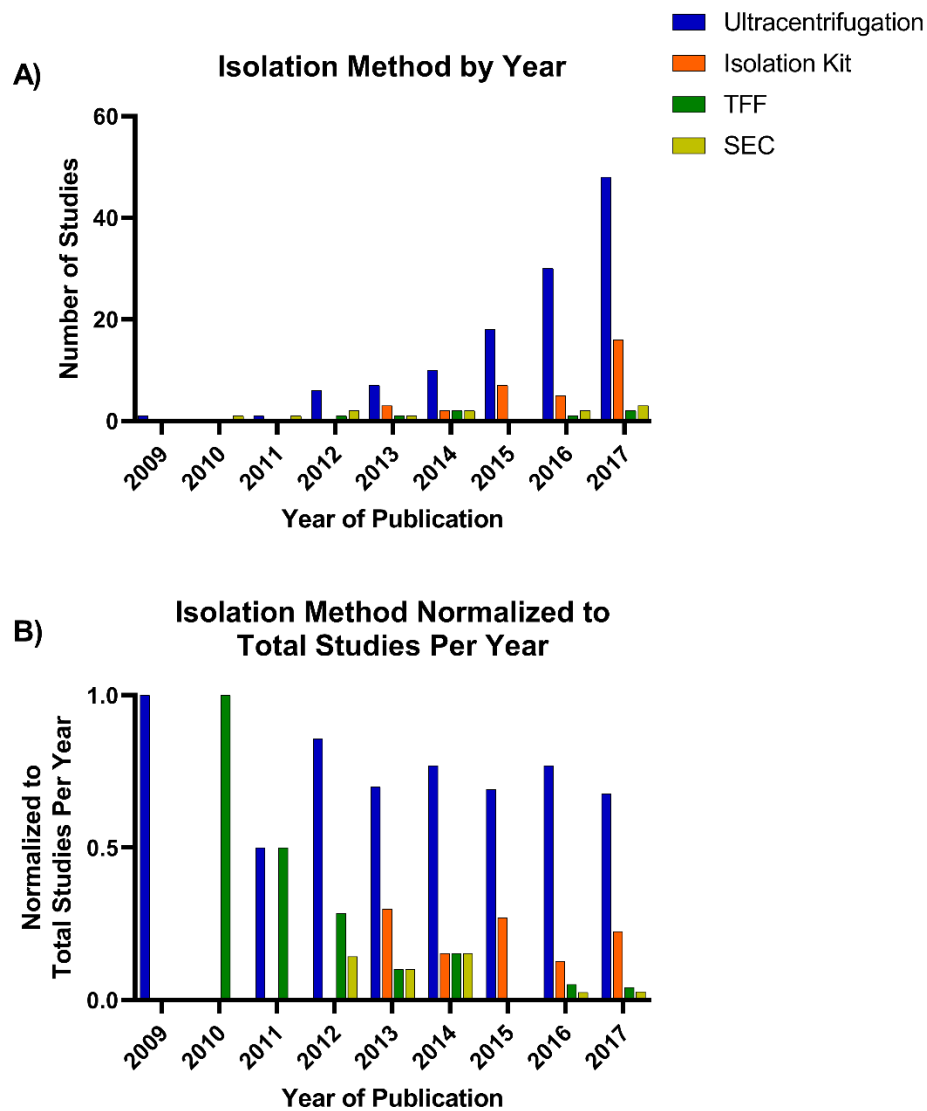
28 or/19-27

29 18 and 28

30 conference abstract.pt.

31 29 not 30

Supplemental File B8 – EV Isolation Method by Year



Supplemental File B8 (A) Isolation method used to generate MSC-EVs depicted by year. (B) MSC-EV isolation method normalized to total number of studies per year, which indicates that ultracentrifugation is consistently the most utilized approach for EV enrichment.

Appendix C – Supplemental Material for Chapter 4

Supplemental File C1 – PRISMA 2020 Checklist

Section and Topic	Item #	Checklist item	Location where items reported
TITLE			
Title	1	Identify the report as a systematic review.	Page 1
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	Pages 2-3
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Pages 4-5
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Page 5
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Pages 6-7
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Page 7
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Page 7, Supp. File 2
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Pages 7-8, Figure 1
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Pages 7-8
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (e.g. for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Pages 6-8
	10b	List and define all other variables for which data were sought (e.g. participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	Pages 6-8
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Page 8
Effect measures	12	Specify for each outcome the effect measure(s) (e.g. risk ratio, mean difference) used in the synthesis or presentation of results.	Pages 9-10
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (e.g. tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Pages 7-10

	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Pages 9-10
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Pages 9-10
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Pages 9-10
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (e.g. subgroup analysis, meta-regression).	Pages 9-10
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	N/A
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Page 9

Section and Topic	Item #	Checklist item	Location where item is reported
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	N/A
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Page 10, Figure 1
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded, and explain why they were excluded.	Figure 1
Study characteristics	17	Cite each included study and present its characteristics.	Page 10, Table 1
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	-18, Figure 11
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (e.g. confidence/credible interval), ideally using structured tables or plots.	Pages 12-15 Figures 2-9
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	Page 10-18
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (e.g. confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	Pages 12-15
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	Pages 12-15
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	N/A

Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	Pages 17-18
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	N/A
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Pages 18-21
	23b	Discuss any limitations of the evidence included in the review.	Page 20
	23c	Discuss any limitations of the review processes used.	Page 20
	23d	Discuss implications of the results for practice, policy, and future research.	Pages 18-21
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	Page 6
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Page 6
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	N/A
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	Page 1, 21-22
Competing interests	26	Declare any competing interests of review authors.	Page 22
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	Page 21

From: Page MJ, McKenzie JE, Bossuyt PM, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi:10.1136/bmj.n71

For more information, visit: www.prisma-statement.org

Supplemental File C2 – Systematic Review Search Strategies

Supplemental File B2.1

Search Strategy from Inception Until March 6, 2019

Database: Embase Classic+Embase, Ovid MEDLINE(R) ALL

Search Strategy:

-
- 1 Mesenchymal Stromal Cells/
 - 2 Mesenchymal Stem Cell Transplantation/
 - 3 Multipotent Stem Cells/
 - 4 (MSC or MSCs or ADMSC or ADMSCs or BM-MSC or BM-MSCs or BMD-MSC or BMD-MSCs or BMDMSC or BMDMSCs).tw,kw.
 - 5 (mesenchymal adj5 (cell* or stem or stromal or progenitor* or multipotent or bone marrow or adipose or placenta*)).tw.
 - 6 (mesenchymal and (cell* or stem or stromal or progenitor or multipotent or bone marrow or adipose or placenta*)).kw.
 - 7 ((multipotent or multi-potent) adj (stroma* cell* or stem cell*)).tw.
 - 8 ((multipotent or multi-potent) and (stroma* cell* or stem cell*)).kw.
 - 9 (colony-forming adj2 fibroblast*).tw.
 - 10 marrow stroma* cell*.tw.
 - 11 Mesoderm/cy
 - 12 or/1-11
 - 13 Exosomes/ or Extracellular Vesicles/
 - 14 exosom*.tw,kw.
 - 15 Cell-Derived Microparticles/
 - 16 (microvesicle* or micro vesicle*).tw,kw.
 - 17 ((cell or cells or extracellular) adj2 vesicle*).tw.
 - 18 microparticle*.tw,kw.
 - 19 ((cell or cells or extracellular) and vesicle*).kw.
 - 20 or/13-19
 - 21 12 and 20
 - 22 exp "animal experimentation"/ or exp "models, animal"/ or exp "invertebrates"/ or "Animals"/ or exp "animal population groups"/ or "chordata"/ or exp "chordata, nonvertebrate"/ or "vertebrates"/ or exp "amphibians"/ or exp "birds"/ or exp "fishes"/ or exp "reptiles"/ or "mammals"/ or "primates"/ or exp "artiodactyla"/ or exp "carnivora"/ or exp "cetacea"/ or exp "chiroptera"/ or exp "elephants"/ or exp "hyraxes"/ or exp "insectivora"/ or exp "lagomorpha"/ or exp "marsupialia"/ or exp "monotremata"/ or exp "perissodactyla"/ or exp "rodentia"/ or exp "scandentia"/ or exp "sirenia"/ or exp "xenarthra"/ or "haplorhini"/ or exp "strepsirhini"/ or exp "platyrrhini"/ or exp "tarsii"/ or "catarrhini"/ or exp "cercopithecidae"/ or exp "hylobatidae"/ or "hominidae"/ or exp "gorilla gorilla"/ or exp "pan paniscus"/ or exp "pan troglodytes"/ or exp "pongo pygmaeus"/ (6252795)
 - 23 (animals or animal or mice or mus or mouse or murine or woodmouse or rats or rat or murinae or muridae or cottonrat or cottonrats or hamster or hamsters or cricetinae or rodentia or rodent or rodents or pigs or pig or swine or swines or piglets or piglet or boar or boars or "sus

scrofa" or ferrets or ferret or polecat or polecats or "mustela putorius" or "guinea pigs" or "guinea pig" or cavia or callithrix or marmoset or marmosets or cebuella or hapale or octodon or chinchilla or chinchillas or gerbillinae or gerbil or gerbils or jird or jirds or merione or meriones or rabbits or rabbit or hares or hare or diptera or flies or fly or dipteral or drosophila or drosophilidae or cats or cat or carus or felis or nematoda or nematode or nematoda or nematode or nematodes or sipunculida or dogs or dog or canine or canines or canis or sheep or sheeps or mouflon or mouflons or ovis or goats or goat or capra or capras or rupicapra or chamois or haplorhini or monkey or monkeys or anthropoidea or anthropoids or saguinus or tamarin or tamarins or leontopithecus or hominidae or ape or apes or pan or paniscus or "pan paniscus" or bonobo or bonobos or troglodytes or "pan troglodytes" or gibbon or gibbons or siamang or siamangs or nomascus or symphalangus or chimpanzee or chimpanzees or prosimians or "bush baby" or prosimian or bush babies or galagos or galago or pongidae or gorilla or gorillas or pongo or pygmaeus or "pongo pygmaeus" or orangutans or pygmaeus or lemur or lemurs or lemuridae or horse or horses or pongo or equus or cow or calf or bull or chicken or chickens or gallus or quail or bird or birds or quails or poultry or poultries or fowl or fowls or reptile or reptilia or reptiles or snakes or snake or lizard or lizards or alligator or alligators or crocodile or crocodiles or turtle or turtles or amphibian or amphibians or amphibia or frog or frogs or bombina or salientia or toad or toads or "epidalea calamita" or salamander or salamanders or eel or eels or fish or fishes or pisces or catfish or catfishes or siluriformes or arius or heteropneustes or sheatfish or perch or perches or percidae or perca or trout or trouts or char or chars or salvelinus or "fathead minnow" or minnow or cyprinidae or carps or carp or zebrafish or zebrafishes or goldfish or goldfishes or guppy or guppies or chub or chubs or tinca or barbels or barbus or pimephales or promelas or "poecilia reticulata" or mullet or mullets or seahorse or seahorses or mugil curema or atlantic cod or shark or sharks or catshark or anguilla or salmonid or salmonids or whitefish or whitefishes or salmon or salmons or sole or solea or "sea lamprey" or lamprey or lampreys or pumpkinseed or sunfish or sunfishes or tilapia or tilapias or turbot or turbots or flatfish or flatfishes or sciuridae or squirrel or squirrels or chipmunk or chipmunks or suslik or susliks or vole or voles or lemming or lemmings or muskrat or muskrats or lemmus or otter or otters or marten or martens or martes or weasel or badger or badgers or ermine or mink or minks or sable or sables or gulo or gulos or wolverine or wolverines or minks or mustela or llama or llamas or alpaca or alpacas or camelid or camelids or guanaco or guanacos or chiroptera or chiropteras or bat or bats or fox or foxes or iguana or iguanas or xenopus laevis or parakeet or parakeets or parrot or parrots or donkey or donkeys or mule or mules or zebra or zebras or shrew or shrews or bison or bisons or buffalo or buffaloes or deer or deers or bear or bears or panda or pandas or "wild hog" or "wild boar" or fitchew or fitch or beaver or beavers or jerboa or jerboas or capybara or capybaras).tw.

- 24 Drug Evaluation, Preclinical/
- 25 (preclinic* or pre clinic*).tw,kw.
- 26 22 or 23 or 24 or 25
- 27 21 and 26

Database: Embase Classic+Embase
Search Strategy:

-
- 1 exp mesenchymal stem cell/
 - 2 exp mesenchymal stem cell transplantation/
 - 3 exp mesenchymal stroma cell/
 - 4 (MSC or MSCs or ADMSC or ADMSCs or BM-MSC or BM-MSCs or BMD-MSC or BMD-MSCs or BMDMSC or BMDMSCs or BMSC or BMSCs or HBMSC or HBMSCs or ABMSC or ABMSCs or MAPC or MAPCs).tw.
 - 5 (((multipotent or multi-potent) adj3 (stem or stroma\$1 or progenitor*)) and (cell\$1 or cell-induced)).tw.
 - 6 multipotent stem cell/
 - 7 (mesenchymal adj5 (cell* or stem or stroma* or progenitor* or multipotent or bone marrow or adipose or placenta*)).tw.
 - 8 (colony-forming adj2 fibroblast*).tw.
 - 9 (marrow stroma* adj2 cell*).tw.
 - 10 or/1-9
 - 11 exosome/
 - 12 exosom*.tw.
 - 13 (microvesicle* or micro vesicle*).tw.
 - 14 microparticle*.tw.
 - 15 ((cell or cells or extracellular) adj2 vesicle*).tw.
 - 16 exp membrane microparticle/
 - 17 or/11-16
 - 18 10 and 17
 - 19 exp animal experiment/ or exp animal model/ or exp experimental animal/ or exp transgenic animal/ or exp male animal/ or exp female animal/ or exp juvenile animal/ or animal/ or chordata/ or vertebrate/
 - 20 tetrapod/ or exp fish/ or amniote/ or exp amphibia/ or mammal/ or exp reptile/ or exp sauropsid/ or therian/ or exp monotremate/ or placental mammals/ or exp marsupial/ or Euarchontoglires/ or exp Afrotheria/ or exp Boreoeutheria/ or exp Laurasiatheria/ or exp Xenarthra/ or primate/ or exp Dermoptera/ or exp Glires/ or exp Scandentia/ or Haplorhini/ or exp prosimian/ or simian/ or exp tarsiiiform/ or Catarrhini/ or exp Platyrrhini/ or ape/ or exp Cercopithecidae/ or hominid/ or exp hylobatidae/ or exp chimpanzee/ or exp gorilla/ or exp orang utan/
 - 21 (preclinic* or pre clinic*).tw.
 - 22 (animal or animals or pisces or fish or fishes or catfish or catfishes or sheatfish or silurus or arius or heteropneustes or clarias or gariepinus or fathead minnow or fathead minnows or pimephales or promelas or cichlidae or trout or trouts or char or chars or salvelinus or salmo or oncorhynchus or guppy or guppies or millionfish or poecilia or goldfish or goldfishes or carassius or auratus or mullet or mullets or mugil or curema or shark or sharks or cod or cods or gadus or morhua or carp or carps or cyprinus or carpio or killifish or eel or eels or anguilla or zander or sander or lucioperca or stizostedion or turbot or turbots or psetta or flatfish or flatfishes or plaice or pleuronectes or platessa or tilapia or tilapias).tw.
 - 23 (oreochromis or sarotherodon or common sole or dover sole or solea or zebrafish or zebrafishes or danio or rerio or seabass or dicentrarchus or labrax or morone or lamprey or

lampreys or petromyzon or pumpkinseed or pumpkinseeds or lepomis or gibbosus or herring or clupea or harengus or amphibia or amphibian or amphibians or anura or salientia or frog or frogs or rana or toad or toads or bufo or xenopus or laevis or bombina or epidalea or calamita or salamander or salamanders or newt or newts or triturus or reptilia or reptile or reptiles or bearded dragon or pogona or vitticeps or iguana or iguanas or lizard).tw.

24 (lizards or anguis fragilis or turtle or turtles or snakes or snake or aves or bird or birds or quail or quails or coturnix or bobwhite or colinus or virginianus or poultry or poultries or fowl or fowls or chicken or chickens or gallus or zebra finch or taeniopygia or guttata or canary or canaries or serinus or canaria or parakeet or parakeets or grasskeet or parrot or parrots or psittacine or psittacines or shelduck or tadorna or goose or geese or branta or leucopsis or woodlark or lullula or flycatcher or ficedula or hypoleuca or dove or doves or geopelia or cuneata or duck or ducks or greylag or graylag or anser or harrier or circus pygargus or red knot or great knot or calidris or canutus or godwit or limosa or lapponica or meleagris or gallopavo or jackdaw or corvus or monedula or ruff or philomachus or pugnax or lapwing or peewit or plover or vanellus or swan or cygnus or columbianus or bewickii or gull or chroicocephalus or ridibundus).tw.

25 (albifrons or great tit or parus or aythya or fuligula or streptopelia or risoria or spoonbill or platalea or leucorodia or blackbird or turdus or merula or blue tit or cyanistes or pigeon or pigeons or columba or pintail or anas or starling or sturnus or owl or athene noctua or pochard or ferina or cockatiel or nymphicus or hollandicus or skylark or alauda or tern or sterna or teal or crecca or oystercatcher or haematopus or ostralegus or shrew or shrews or sorex or araneus or crocidura or russula or european mole or talpa or chiroptera or bat or bats or eptesicus or serotinus or myotis or dasycneme or daubentonii or pipistrelle or pipistrellus or cat or cats or felis or catus or feline or dog or dogs or canis or canine or canines or otter or otters or lutra or badger or badgers or meles or fitchew or fitch or foumart or foulmart or ferrets or ferret or polecat or polecats or mustela or putorius or weasel or weasels or fox or foxes or vulpes or common seal or phoca or vitulina or grey seal or halichoerus or horse or horses or equus or equine or equidae or donkey or donkeys or mule or mules or pig or pigs or swine or swines or hog or hogs or boar or boars or porcine or piglet or piglets or sus or scrofa or llama or llamas or lama or glama or deer or deers or cervus or elaphus or cow or cows or bos taurus or bos indicus or bovine or bull or bulls).tw.

26 (cattle or bison or bisons or sheep or sheeps or ovis aries or ovine or lamb or lambs or mouflon or mouflons or goat or goats or capra or caprine or chamois or rupicapra or leporidae or lagomorpha or lagomorph or rabbit or rabbits or oryctolagus or cuniculus or laprine or hares or lepus or rodentia or rodent or rodents or murinae or mouse or mice or mus or musculus or murine or woodmouse or apodemus or rat or rats or rattus or norvegicus or guinea pig or guinea pigs or cavia or porcellus or hamster or hamsters or mesocricetus or cricetulus or cricetus or gerbil or gerbils or jird or jirds or meriones or unguiculatus or jerboa or jerboas or jaculus or chinchilla or chinchillas or beaver or beavers or castor fiber or castor canadensis or sciuridae or squirrel or squirrels or sciurus or chipmunk or chipmunks or marmot or marmots or marmota or suslik or susliks or spermophilus or cynomys or cottonrat or cottonrats or sigmodon or vole or voles or microtus or myodes or glareolus or primate).tw.

27 (primates or prosimian or prosimians or lemur or lemurs or lemuridae or loris or bush baby or bush babies or bushbaby or bushbabies or galago or galagos or anthropoidea or anthropoids or simian or simians or monkey or monkeys or marmoset or marmosets or callithrix or cebuella or tamarin or tamarins or saguinus or leontopithecus or squirrel monkey or squirrel monkeys or

saimiri or night monkey or night monkeys or owl monkey or owl monkeys or douroucoulis or aotus or spider monkey or spider monkeys or ateles or baboon or baboons or papio or rhesus monkey or macaque or macaca or mulatta or cynomolgus or fascicularis or green monkey or green monkeys or chlorocebus or vervet or vervets or pygerythrus or hominoidea or ape or apes or hylobatidae or gibbon or gibbons or siamang or siamangs or nomascus or symphalangus or hominidae or orangutan or orangutans or pongo or chimpanzee or chimpanzees or pan troglodytes or bonobo or bonobos or pan paniscus or gorilla or gorillas or troglodytes).tw.

28 or/19-27

29 18 and 28

30 conference abstract.pt.

31 29 not 30

Supplemental File 2B.2

Search Strategy from March 6, 2019 to August 4, 2020

Database: Embase Classic+Embase, Ovid MEDLINE(R) ALL

Search Strategy:

-
- 1 Mesenchymal Stromal Cells/ (47141)
 - 2 Mesenchymal Stem Cell Transplantation/ (23442)
 - 3 Multipotent Stem Cells/ (8748)
 - 4 (MSC or MSCs or ADMSC or ADMSCs or BM-MSC or BM-MSCs or BMD-MSC or BMD-MSCs or BMDMSC or BMDMSCs).tw,kw. (82683)
 - 5 (mesenchymal adj5 (cell* or stem or stromal or progenitor* or multipotent or bone marrow or adipose or placenta*)).tw. (187129)
 - 6 (mesenchymal and (cell* or stem or stromal or progenitor or multipotent or bone marrow or adipose or placenta*)).kw. (30852)
 - 7 ((multipotent or multi-potent) adj (stroma* cell* or stem cell*)).tw. (4480)
 - 8 ((multipotent or multi-potent) and (stroma* cell* or stem cell*)).kw. (729)
 - 9 (colony-forming adj2 fibroblast*).tw. (1629)
 - 10 marrow stroma* cell*.tw. (17148)
 - 11 Mesoderm/cy (5813)
 - 12 or/1-11 (235330)
 - 13 Exosomes/ or Extracellular Vesicles/ (37062)
 - 14 exosom*.tw,kw. (36154)
 - 15 Cell-Derived Microparticles/ (8299)
 - 16 (microvesicle* or micro vesicle*).tw,kw. (10732)
 - 17 ((cell or cells or extracellular) adj2 vesicle*).tw. (25017)
 - 18 microparticle*.tw,kw. (39690)
 - 19 ((cell or cells or extracellular) and vesicle*).kw. (5382)
 - 20 or/13-19 (101737)
 - 21 12 and 20 (6645)
 - 22 (animals or animal or mice or mus or mouse or murine or woodmouse or rats or rat or murinae or muridae or cottonrat or cottonrats or hamster or hamsters or cricetinae or rodentia or rodent or rodents or pigs or pig or swine or swines or piglets or piglet or boar or boars or "sus scrofa" or ferrets or ferret or polecat or polecats or "mustela putorius" or "guinea pigs" or "guinea pig" or cavia or callithrix or marmoset or marmosets or cebuella or hapale or octodon or chinchilla or chinchillas or gerbillinae or gerbil or gerbils or jird or jirds or merione or meriones or rabbits or rabbit or hares or hare or diptera or flies or fly or dipteral or drosophila or drosophilidae or cats or cat or carus or felis or nematoda or nematode or nematoda or nematode or nematodes or sipunculida or dogs or dog or canine or canines or canis or sheep or sheeps or mouflon or mouflons or ovis or goats or goat or capra or capras or rupicapra or chamois or haplorhini or monkey or monkeys or anthropoidea or anthropoids or saguinus or tamarin or tamarins or leontopithecus or hominidae or ape or apes or pan or paniscus or "pan paniscus" or bonobo or bonobos or troglodytes or "pan troglodytes" or gibbon or gibbons or siamang or siamangs or nomascus or symphalangus or chimpanzee or chimpanzees or prosimians or "bush baby" or prosimian or bush babies or galagos or galago or pongidae or gorilla or gorillas or

pongo or pygmaeus or "pongo pygmaeus" or orangutans or pygmaeus or lemur or lemurs or lemuridae or horse or horses or pongo or equus or cow or calf or bull or chicken or chickens or gallus or quail or bird or birds or quails or poultry or poultries or fowl or fowls or reptile or reptilia or reptiles or snakes or snake or lizard or lizards or alligator or alligators or crocodile or crocodiles or turtle or turtles or amphibian or amphibians or amphibia or frog or frogs or bombina or salientia or toad or toads or "epidalea calamita" or salamander or salamanders or eel or eels or fish or fishes or pisces or catfish or catfishes or siluriformes or arius or heteropneustes or sheatfish or perch or perches or percidae or perca or trout or trouts or char or chars or salvelinus or "fathead minnow" or minnow or cyprinidae or carps or carp or zebrafish or zebrafishes or goldfish or goldfishes or guppy or guppies or chub or chubs or tinca or barbels or barbus or pimephales or promelas or "poecilia reticulata" or mullet or mullets or seahorse or seahorses or mugil curema or atlantic cod or shark or sharks or catshark or anguilla or salmonid or salmonids or whitefish or whitefishes or salmon or salmons or sole or solea or "sea lamprey" or lamprey or lampreys or pumpkinseed or sunfish or sunfishes or tilapia or tilapias or turbot or turbots or flatfish or flatfishes or sciuridae or squirrel or squirrels or chipmunk or chipmunks or suslik or susliks or vole or voles or lemming or lemmings or muskrat or muskrats or lemmus or otter or otters or marten or martens or martes or weasel or badger or badgers or ermine or mink or minks or sable or sables or gulo or gulos or wolverine or wolverines or minks or mustela or llama or llamas or alpaca or alpacas or camelid or camelids or guanaco or guanacos or chiroptera or chiropteras or bat or bats or fox or foxes or iguana or iguanas or xenopus laevis or parakeet or parakeets or parrot or parrots or donkey or donkeys or mule or mules or zebra or zebras or shrew or shrews or bison or bisons or buffalo or buffaloes or deer or deers or bear or bears or panda or pandas or "wild hog" or "wild boar" or fitchew or fitch or beaver or beavers or jerboa or jerboas or capybara or capybaras).tw. (11030514)

23 Drug Evaluation, Preclinical/ (79575)

24 (preclinic* or pre clinic*).tw,kw. (290442)

25 exp "animal experimentation"/ or exp "models, animal"/ or exp "invertebrates"/ or "Animals"/ or exp "animal population groups"/ or "chordata"/ or exp "chordata, nonvertebrate"/ or "vertebrates"/ or exp "amphibians"/ or exp "birds"/ or exp "fishes"/ or exp "reptiles"/ or "mammals"/ or "primates"/ or exp "artiodactyla"/ or exp "carnivora"/ or exp "cetacea"/ or exp "chiroptera"/ or exp "elephants"/ or exp "hyraxes"/ or exp "insectivora"/ or exp "lagomorpha"/ or exp "marsupialia"/ or exp "monotremata"/ or exp "perissodactyla"/ or exp "rodentia"/ or exp "scandentia"/ or exp "sirenia"/ or exp "xenarthra"/ or "haplorhini"/ or exp "strepsirhini"/ or exp "platyrrhini"/ or exp "tarsii"/ or "catarrhini"/ or exp "cercopithecidae"/ or exp "hylobatidae"/ or "hominidae"/ or exp "gorilla gorilla"/ or exp "pan paniscus"/ or exp "pan troglodytes"/ or exp "pongo pygmaeus"/ (35047508)

26 22 or 23 or 24 or 25 (36487721)

27 21 and 26 (5482)

28 exp respiratory tract diseases/ (4073446)

29 (lung or pulmonary or respirator*).tw,kw. (3278236)

30 copd.tw,kw. (136879)

31 bronch*.tw,kw. (611235)

32 or/28-31 (5537909)

33 27 and 32 (770)

34 33 use medall (165)

35 (201903* or 201904* or 201905* or 201906* or 201907* or 201908* or 201909* or 20191* or 2020*).dt. (1932577)

36 34 and 35 (75) Medline

37 exp mesenchymal stem cell/ (97594)

38 exp mesenchymal stem cell transplantation/ (23442)

39 exp mesenchymal stroma cell/ (13575)

40 (MSC or MSCs or ADMSC or ADMSCs or BM-MSC or BM-MSCs or BMD-MSC or BMD-MSCs or BMDMSC or BMDMSCs or BMSC or BMSCs or HBMSC or HBMSCs or ABMSC or ABMSCs or MAPC or MAPCs).tw. (99021)

41 (((multipotent or multi-potent) adj3 (stem or stroma\$1 or progenitor*)) and (cell\$1 or cell-induced)).tw. (15745)

42 multipotent stem cell/ (9063)

43 (mesenchymal adj5 (cell* or stem or stroma* or progenitor* or multipotent or bone marrow or adipose or placenta*)).tw. (187599)

44 (colony-forming adj2 fibroblast*).tw. (1629)

45 (marrow stroma* adj2 cell*).tw. (17959)

46 or/37-45 (244010)

47 exosome/ (27956)

48 exosom*.tw. (33211)

49 (microvesicle* or micro vesicle*).tw. (9612)

50 microparticle*.tw. (38211)

51 ((cell or cells or extracellular) adj2 vesicle*).tw. (25017)

52 exp membrane microparticle/ (7719)

53 or/47-52 (98136)

54 46 and 53 (6923)

55 exp animal experiment/ or exp animal model/ or exp experimental animal/ or exp transgenic animal/ or exp male animal/ or exp female animal/ or exp juvenile animal/ or animal/ or chordata/ or vertebrate/ (11657807)

56 tetrapod/ or exp fish/ or amniote/ or exp amphibia/ or mammal/ or exp reptile/ or exp sauropsid/ or therian/ or exp monotremate/ or placental mammals/ or exp marsupial/ or Euarchontoglires/ or exp Afrotheria/ or exp Boreoeutheria/ or exp Laurasiatheria/ or exp Xenarthra/ or primate/ or exp Dermoptera/ or exp Glires/ or exp Scandentia/ or Haplorhini/ or exp prosimian/ or simian/ or exp tarsiiiform/ or Catarrhini/ or exp Platyrrhini/ or ape/ or exp Cercopithecidae/ or hominid/ or exp hylobatidae/ or exp chimpanzee/ or exp gorilla/ or exp orang utan/ (5999306)

57 (preclinic* or pre clinic*).tw. (287618)

58 (animal or animals or pisces or fish or fishes or catfish or catfishes or sheatfish or silurus or arius or heteropneustes or clarias or gariepinus or fathead minnow or fathead minnows or pimephales or promelas or cichlidae or trout or trouts or char or chars or salvelinus or salmo or oncorhynchus or guppy or guppies or millionfish or poecilia or goldfish or goldfishes or carassius or auratus or mullet or mullets or mugil or curema or shark or sharks or cod or cods or gadus or morhua or carp or carps or cyprinus or carpio or killifish or eel or eels or anguilla or zander or sander or lucioperca or stizostedion or turbot or turbots or psetta or flatfish or flatfishes or plaice or pleuronectes or platessa or tilapia or tilapias).tw. (3022465)

59 (oreochromis or sarotherodon or common sole or dover sole or solea or zebrafish or zebrafishes or danio or rerio or seabass or dicentrarchus or labrax or morone or lamprey or

lampreys or petromyzon or pumpkinseed or pumpkinseeds or lepomis or gibbosus or herring or clupea or harengus or amphibia or amphibian or amphibians or anura or salientia or frog or frogs or rana or toad or toads or bufo or xenopus or laevis or bombina or epidalea or calamita or salamander or salamanders or newt or newts or triturus or reptilia or reptile or reptiles or bearded dragon or pogona or vitticeps or iguana or iguanas or lizard).tw. (349879)

60 (lizards or anguis fragilis or turtle or turtles or snakes or snake or aves or bird or birds or quail or quails or coturnix or bobwhite or colinus or virginianus or poultry or poultries or fowl or fowls or chicken or chickens or gallus or zebra finch or taeniopygia or guttata or canary or canaries or serinus or canaria or parakeet or parakeets or grasskeet or parrot or parrots or psittacine or psittacines or shelduck or tadorna or goose or geese or branta or leucopsis or woodlark or lullula or flycatcher or ficedula or hypoleuca or dove or doves or geopelia or cuneata or duck or ducks or greylag or graylag or anser or harrier or circus pygargus or red knot or great knot or calidris or canutus or godwit or limosa or lapponica or meleagris or gallopavo or jackdaw or corvus or monedula or ruff or philomachus or pugnax or lapwing or peewit or plover or vanellus or swan or cygnus or columbianus or bewickii or gull or chroicocephalus or ridibundus).tw. (515842)

61 (albifrons or great tit or parus or aythya or fuligula or streptopelia or risoria or spoonbill or platalea or leucorodia or blackbird or turdus or merula or blue tit or cyanistes or pigeon or pigeons or columba or pintail or anas or starling or sturnus or owl or athene noctua or pochard or ferina or cockatiel or nymphicus or hollandicus or skylark or alauda or tern or sterna or teal or crecca or oystercatcher or haematopus or ostralegus or shrew or shrews or sorex or araneus or crocidura or russula or european mole or talpa or chiroptera or bat or bats or eptesicus or serotinus or myotis or dasycneme or daubentonii or pipistrelle or pipistrellus or cat or cats or felis or catus or feline or dog or dogs or canis or canine or canines or otter or otters or lutra or badger or badgers or meles or fitchew or fitch or foumart or foulmart or ferrets or ferret or polecat or polecats or mustela or putorius or weasel or weasels or fox or foxes or vulpes or common seal or phoca or vitulina or grey seal or halichoerus or horse or horses or equus or equine or equidae or donkey or donkeys or mule or mules or pig or pigs or swine or swines or hog or hogs or boar or boars or porcine or piglet or piglets or sus or scrofa or llama or llamas or lama or glama or deer or deers or cervus or elaphus or cow or cows or bos taurus or bos indicus or bovine or bull or bulls).tw. (2409314)

62 (cattle or bison or bisons or sheep or sheeps or ovis aries or ovine or lamb or lambs or mouflon or mouflons or goat or goats or capra or caprine or chamois or rupicapra or leporidae or lagomorpha or lagomorph or rabbit or rabbits or oryctolagus or cuniculus or laprine or hares or lepus or rodentia or rodent or rodents or murinae or mouse or mice or mus or musculus or murine or woodmouse or apodemus or rat or rats or rattus or norvegicus or guinea pig or guinea pigs or cavia or porcellus or hamster or hamsters or mesocricetus or cricetulus or cricetus or gerbil or gerbils or jird or jirds or meriones or unguiculatus or jerboa or jerboas or jaculus or chinchilla or chinchillas or beaver or beavers or castor fiber or castor canadensis or sciuridae or squirrel or squirrels or sciurus or chipmunk or chipmunks or marmot or marmots or marmota or suslik or susliks or spermophilus or cynomys or cottonrat or cottonrats or sigmodon or vole or voles or microtus or myodes or glareolus or primate).tw. (7436455)

63 (primates or prosimian or prosimians or lemur or lemurs or lemuridae or loris or bush baby or bush babies or bushbaby or bushbabies or galago or galagos or anthropoidea or anthropoids or simian or simians or monkey or monkeys or marmoset or marmosets or callithrix or cebuella or tamarin or tamarins or saguinus or leontopithecus or squirrel monkey or squirrel monkeys or

saimiri or night monkey or night monkeys or owl monkey or owl monkeys or douroucoulis or aotus or spider monkey or spider monkeys or ateles or baboon or baboons or papio or rhesus monkey or macaque or macaca or mulatta or cynomolgus or fascicularis or green monkey or green monkeys or chlorocebus or vervet or vervets or pygerythrus or hominoidea or ape or apes or hylobatidae or gibbon or gibbons or siamang or siamangs or nomascus or symphalangus or hominidae or orangutan or orangutans or pongo or chimpanzee or chimpanzees or pan troglodytes or bonobo or bonobos or pan paniscus or gorilla or gorillas or troglodytes).tw. (375276)

64 or/55-63 (15670861)

65 54 and 64 (3966)

66 conference abstract.pt. (3841901)

67 65 not 66 (3199)

68 exp respiratory tract disease/ (4073446)

69 (lung or pulmonary or respirator*).tw. (3243475)

70 copd.tw. (133768)

71 bronch*.tw. (597638)

72 68 or 69 or 70 or 71 (5520675)

73 67 and 72 (391)

74 73 use emczd (230)

75 (201903* or 201904* or 201905* or 201906* or 201907* or 201908* or 201909* or 20191* or 2020*).dc. (2912261)

76 74 and 75 (125) Embase

77 36 or 76 (200)

78 remove duplicates from 77 (129)

79 78 use medall (73)

80 78 use emczd (56)

Supplemental File C3 – Study Characteristics, EV Methodology and Interventional Traits

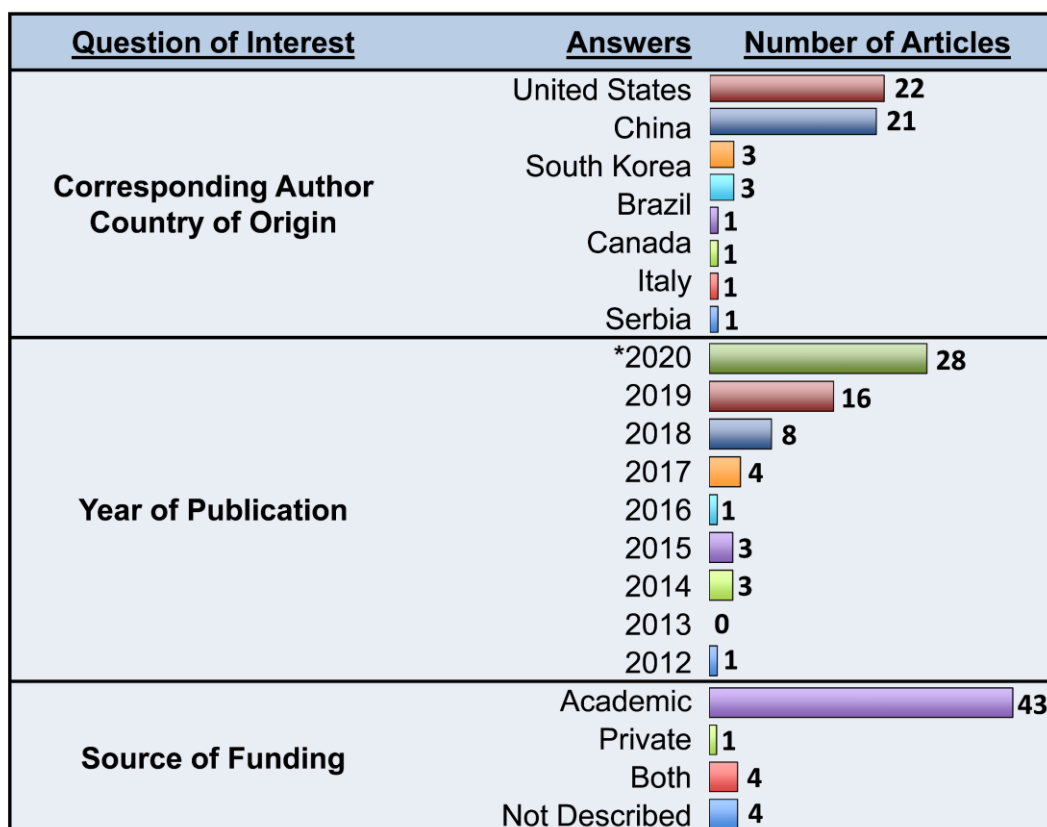


Figure S3.1. Overview of general study characteristics including country of origin, year of publication and source of funding from all 52 lung disease articles. *The number of MSC-EV lung studies published in 2020 is extrapolated from the total number of included studies until August 4, 2020 (the end of our search date).

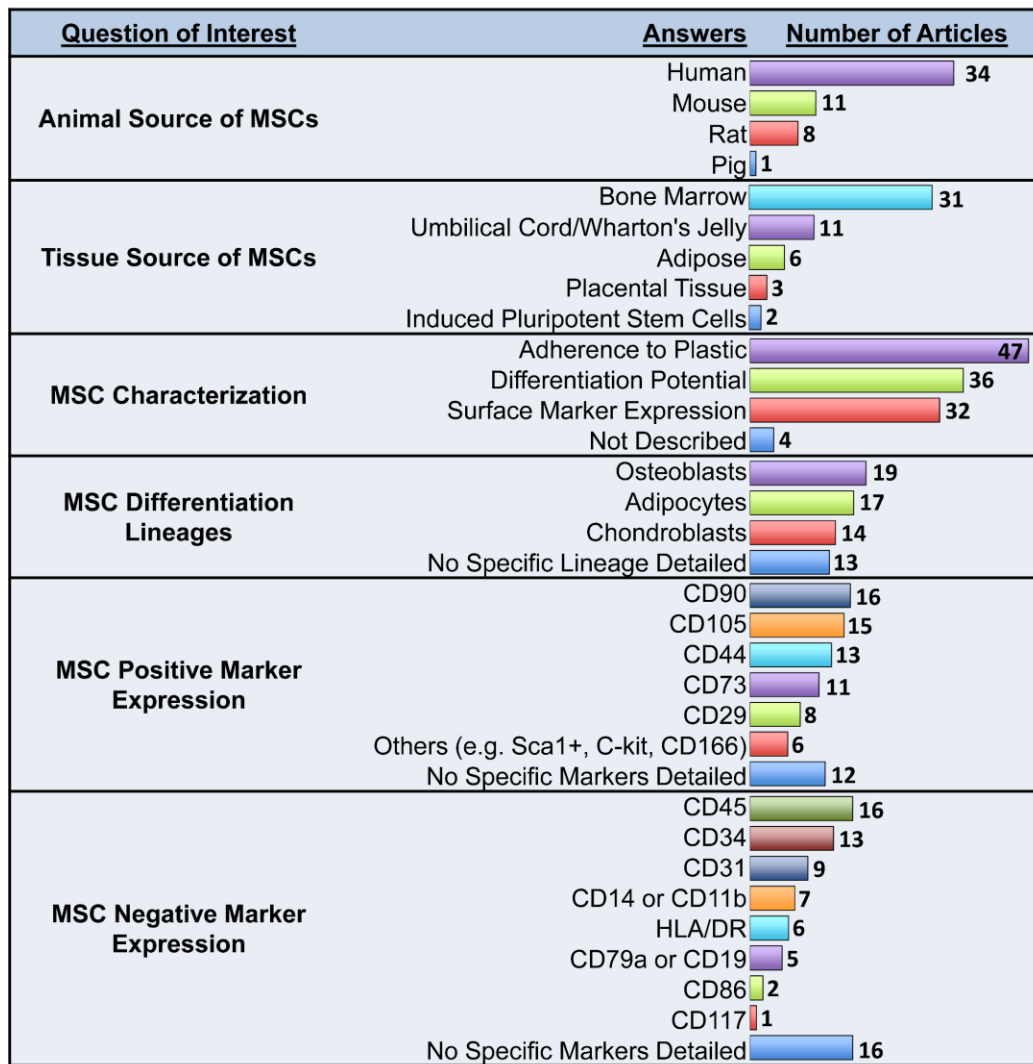


Figure S3.2. Intervention details of administered extracellular vesicles (EVs). The tissue and animal source of mesenchymal stromal cells (MSCs) were extracted from 52 included articles. Studies were assessed for adherence to the MSC characterization standards set forth by the International Society for Cellular Therapy (ISCT). Articles may have utilized EVs from more than one animal and/or tissue source.

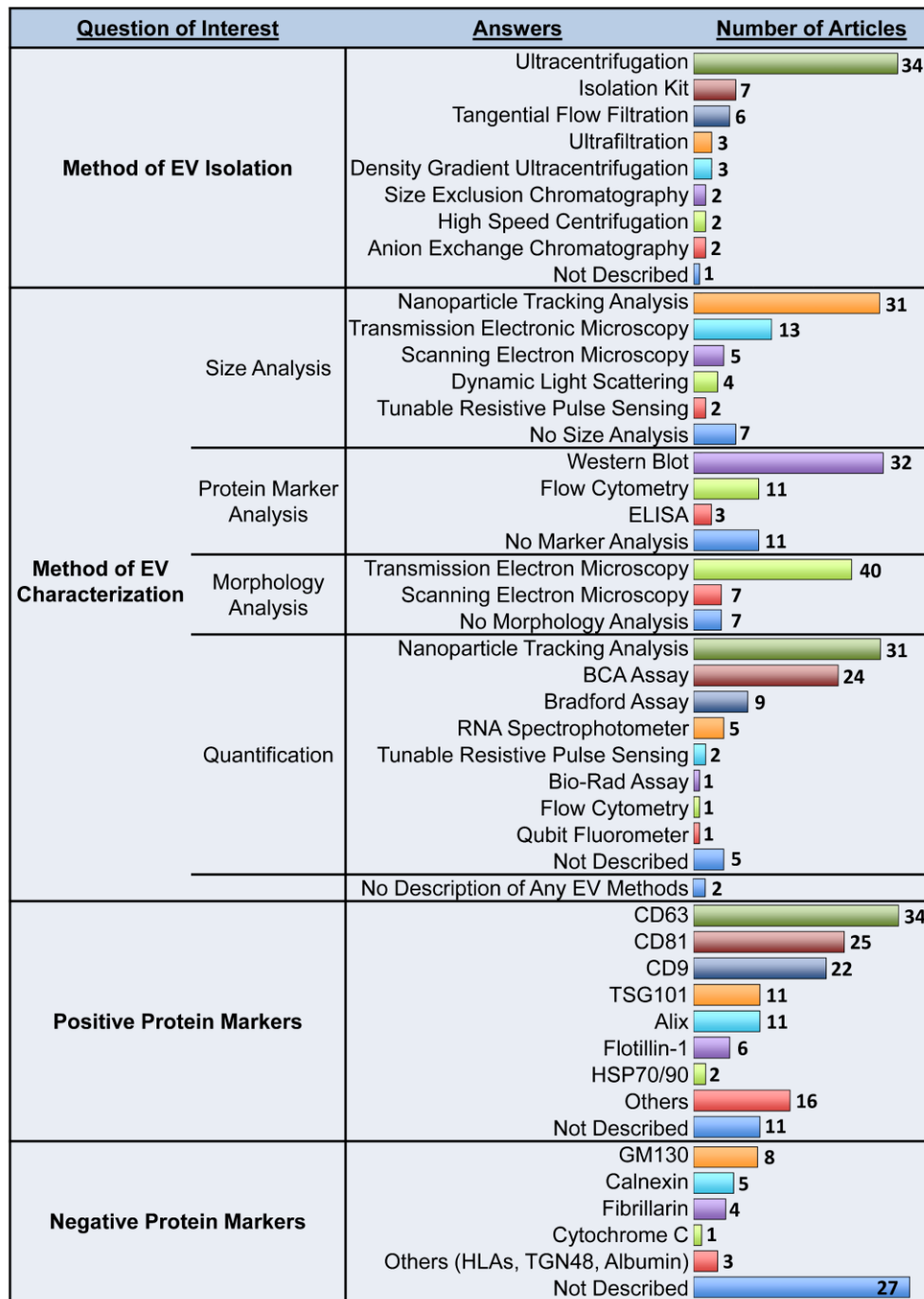


Figure S3.3. Overview of methods of extracellular vesicle (EV) isolation and characterization from 52 lung disease articles. The specific positive and negative protein markers being used were recorded. Studies may have incorporated more than one method within each domain.

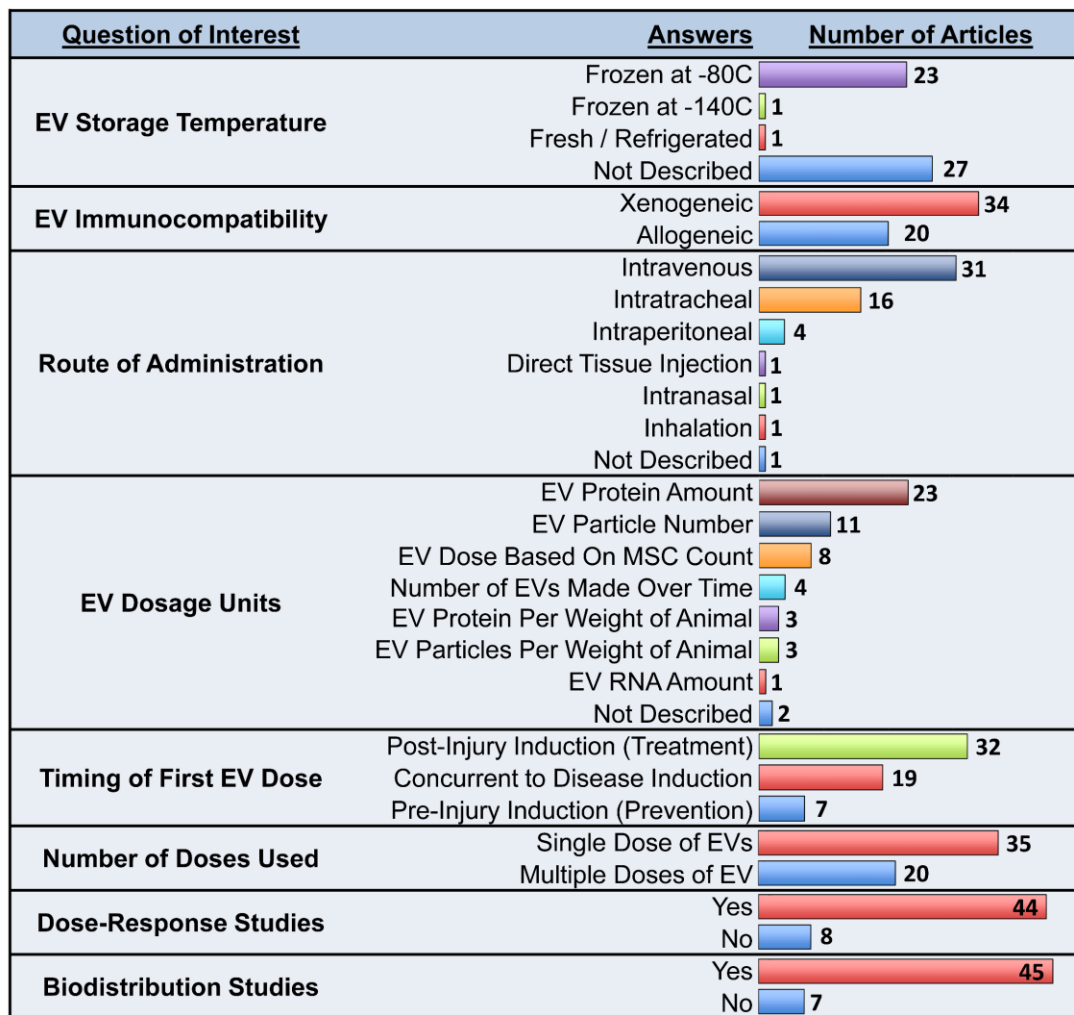


Figure S3.4. Dosage regimen of administered mesenchymal stromal cell-derived extracellular vesicles (MSC-EVs). Details pertaining to route of administration, units of dose used, number of doses, timing of administration, biodistribution analysis and prevalence of dose-response studies was extracted from 52 articles. Studies may have incorporated more than one dosage regimen.

Supplemental File C4 – Meta-Analyses of Secondary Outcomes

Secondary Outcome: Mortality

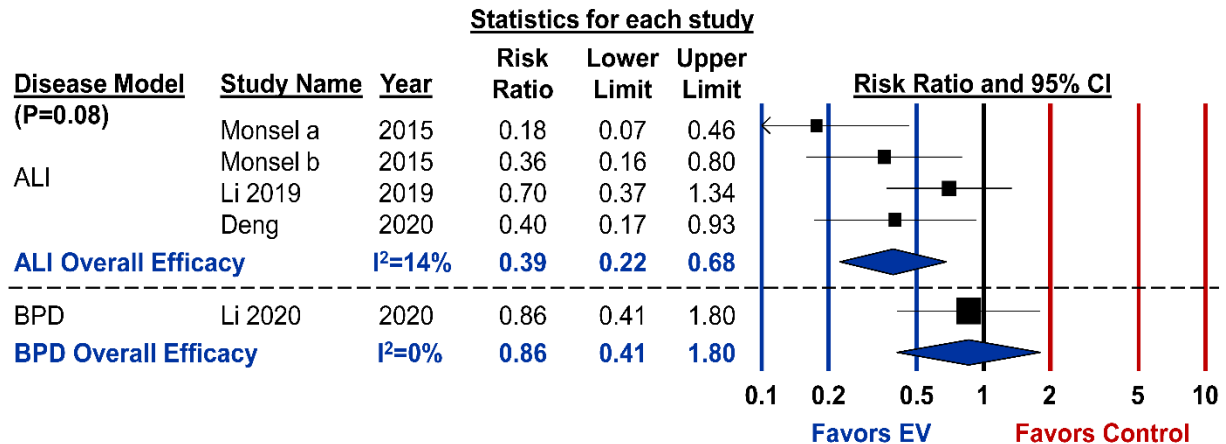


Figure S4.1. Meta-analysis for all included studies of acute lung injury (ALI) and bronchopulmonary dysplasia (BPD) that reported the outcome of mortality. Data is presented as a forest plot with risk ratios and 95% confidence intervals. Effect sizes <1 favors EV treatment and >1 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect on mortality from all studies combined. I^2 value represents the statistical heterogeneity.

Secondary Outcome: BALF Neutrophil

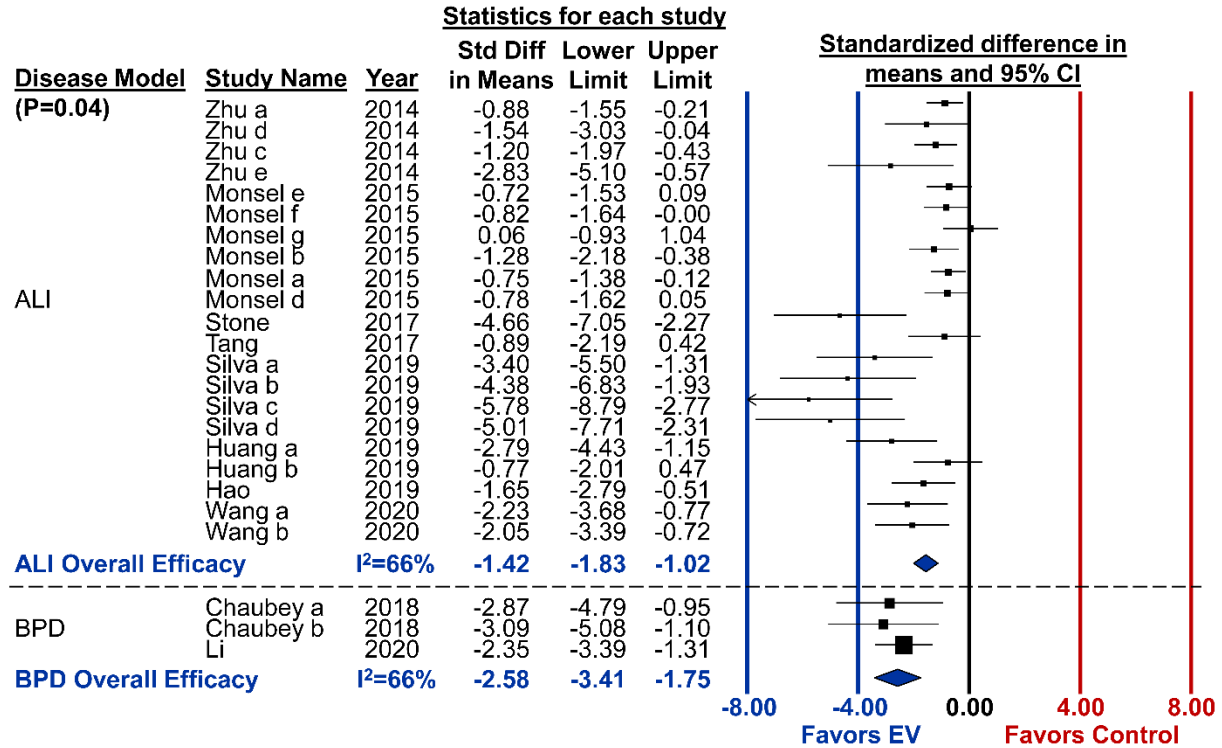


Figure S4.2. Meta-analysis for all included studies of acute lung injury (ALI) and bronchopulmonary dysplasia (BPD) that reported the outcome of bronchoalveolar lavage fluid (BALF) neutrophil. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies combined. I² value represents the statistical heterogeneity.

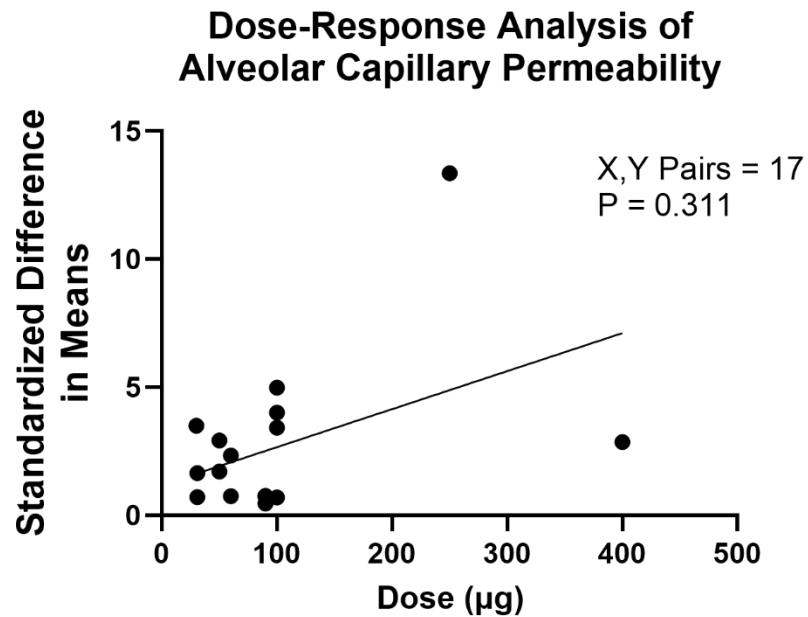


Figure S4.3. Dose-response analysis of acute lung injury studies reporting alveolar capillary permeability as an outcome (N=17 studies). The dose of administered extracellular vesicles (EVs) and their corresponding standardized difference in mean value were assessed for correlation. Despite many studies administering similar doses of EVs, there was no significant correlation observed between dose and effect on outcome.

Secondary Outcome: Fulton's Index

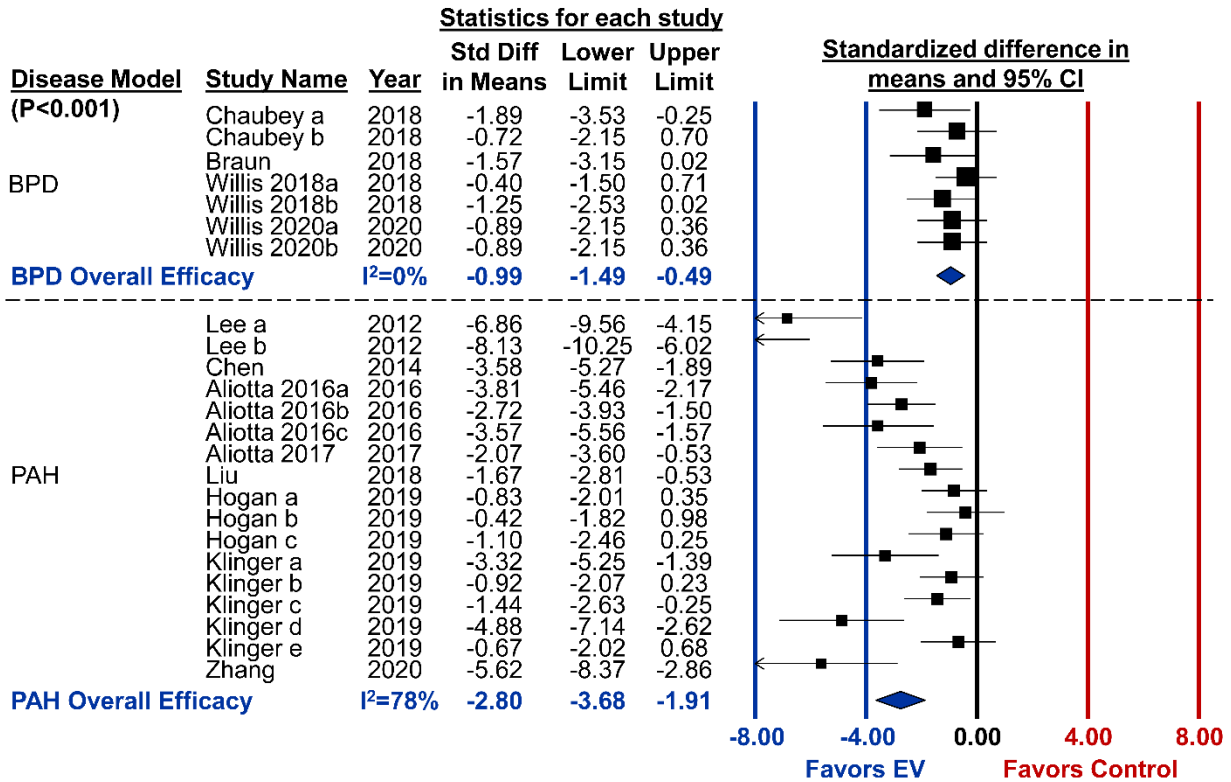


Figure S4.4. Meta-analysis for all included studies of bronchopulmonary dysplasia (BPD) and pulmonary arterial hypertension (PAH) that reported the outcome of Fulton's Index, a measure of right ventricular hypertrophy. Data is presented as a forest plot with standardized mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The 'Overall Efficacy' represents a pooled estimate of MSC-EV effect from all studies combined. I² value represents the statistical heterogeneity.

Secondary Outcome: RVSP for BPD

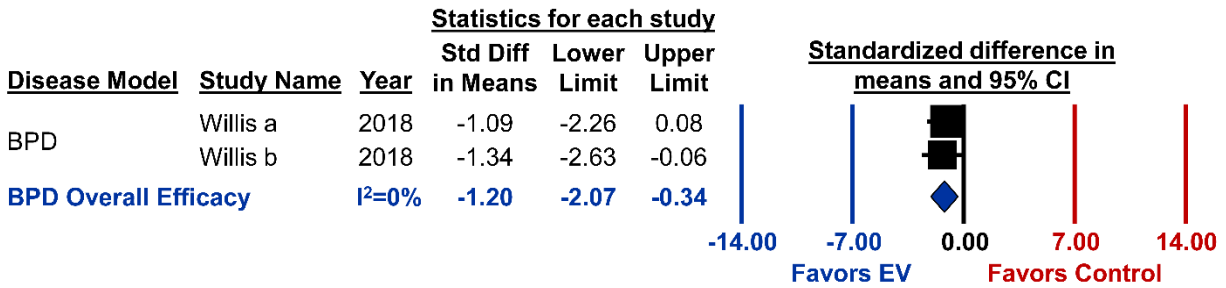


Figure S4.5. Meta-analysis for all included studies of bronchopulmonary dysplasia (BPD) that reported the outcome of right ventricular systolic pressure (RVSP). Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies combined. I^2 value represents the statistical heterogeneity.

Supplemental File C5 – Subgroup Analyses of Outcomes

Subgroup Analysis of Acute Lung Injury – Histological Lung Injury Score:

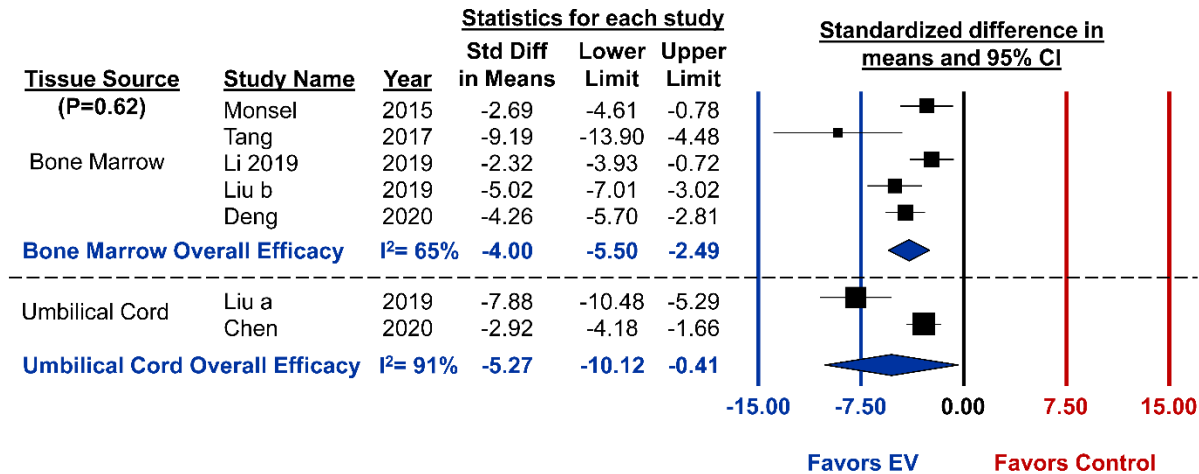


Figure S5.1. Subgroup analysis by MSC tissue source for studies of acute lung injury (ALI) that reported the outcome of lung injury score. Data is presented as a forest plot with standardized mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity.

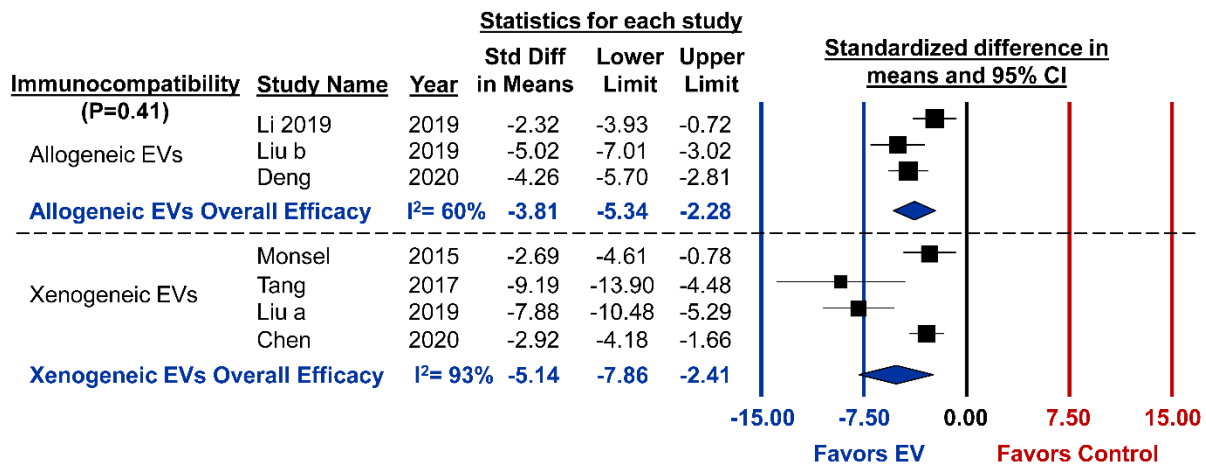


Figure S5.2. Subgroup analysis by EV immunocompatibility for studies of acute lung injury (ALI) that reported the outcome of lung injury score. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity.

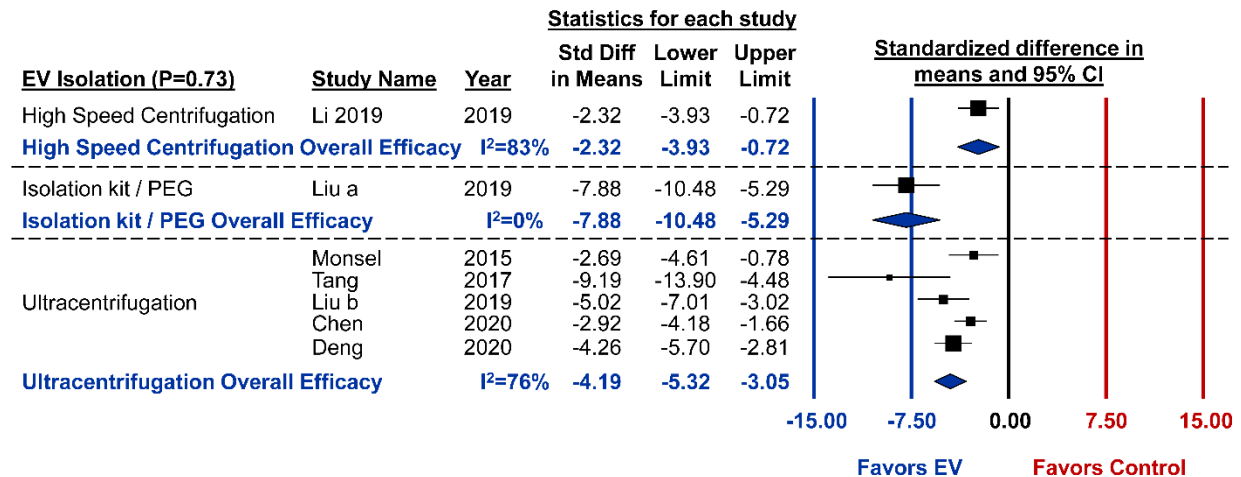


Figure S5.3. Subgroup analysis by EV isolation technique for studies of acute lung injury (ALI) that reported the outcome of lung injury score. Data is presented as a forest plot with standardized mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity.

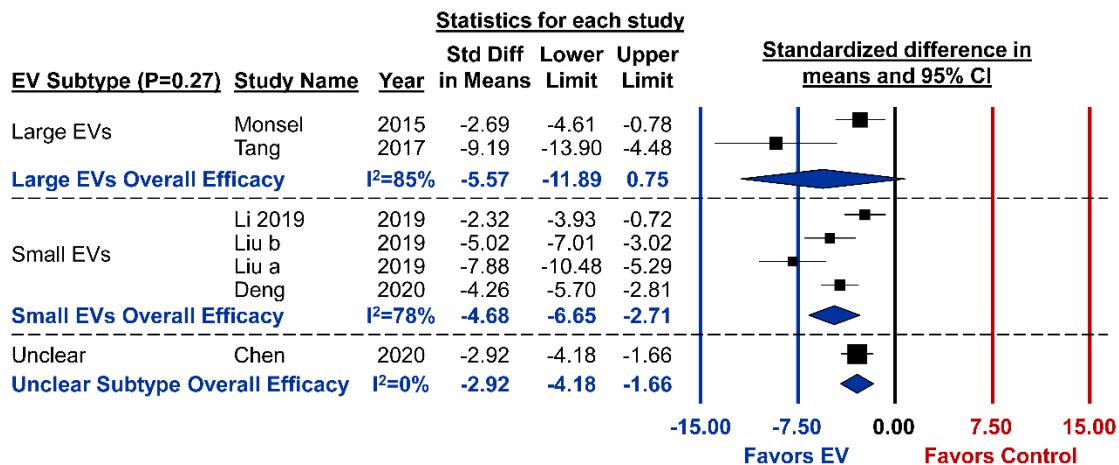


Figure S5.4. Subgroup analysis by EV subtype for studies of acute lung injury (ALI) that reported the outcome of lung injury score. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity.

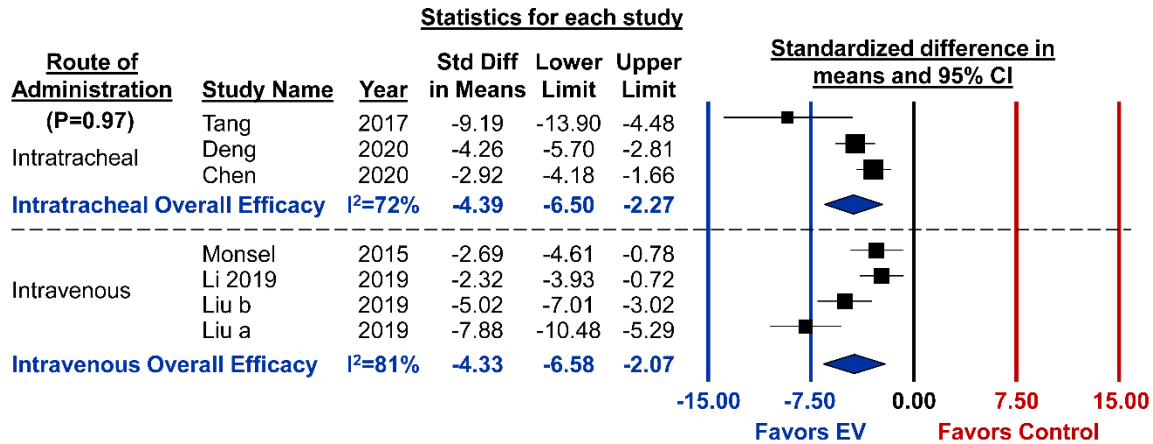


Figure S5.5. Subgroup analysis by route of administration for studies of acute lung injury (ALI) that reported the outcome of lung injury score. Data is presented as a forest plot with standardized mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I^2 value represents the statistical heterogeneity.

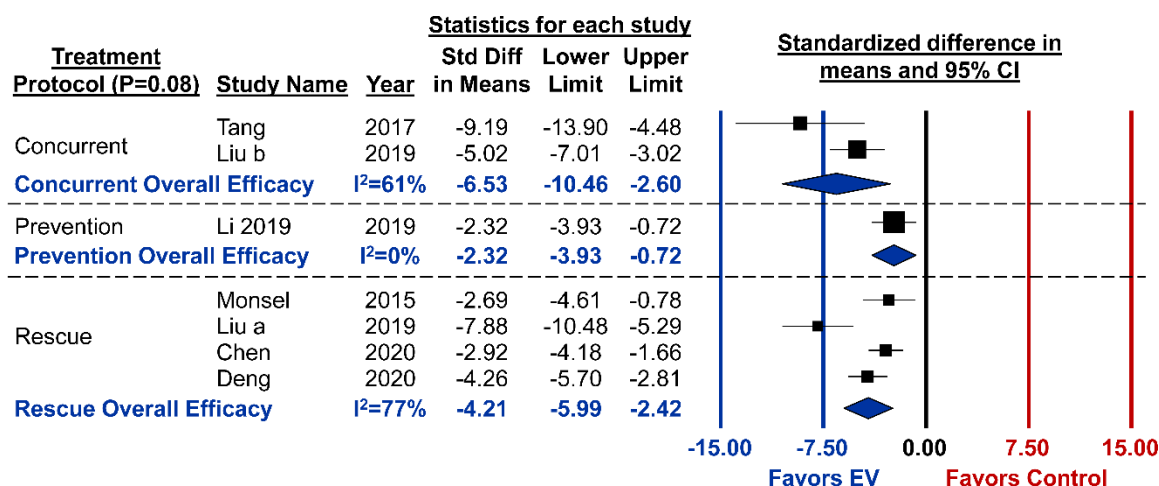


Figure S5.6. Subgroup analysis by timing of treatment for studies of acute lung injury (ALI) that reported the outcome of lung injury score. Data is presented as a forest plot with standardized mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I^2 value represents the statistical heterogeneity.

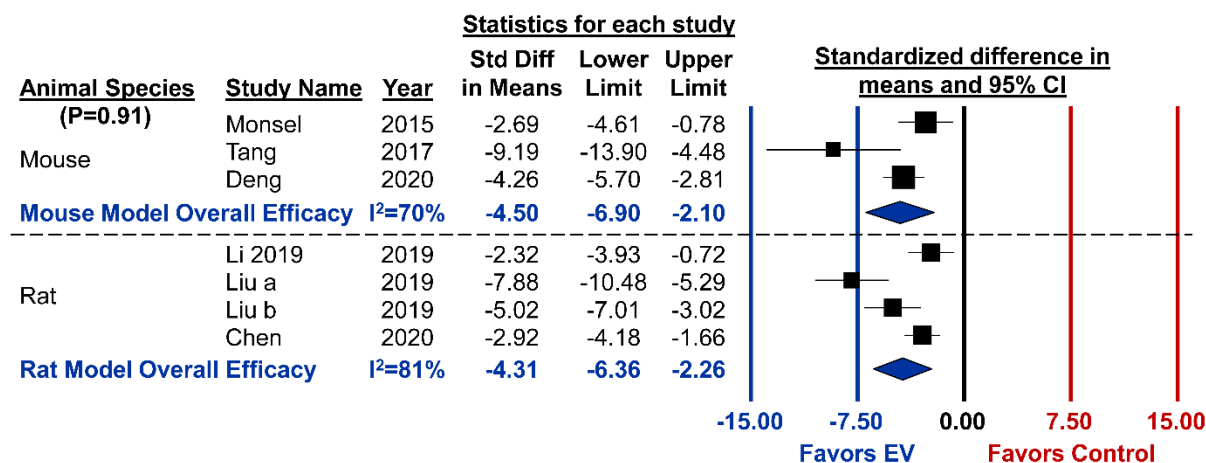


Figure S5.7. Subgroup analysis by species of animal model for studies of acute lung injury (ALI) that reported the outcome of lung injury score. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity.

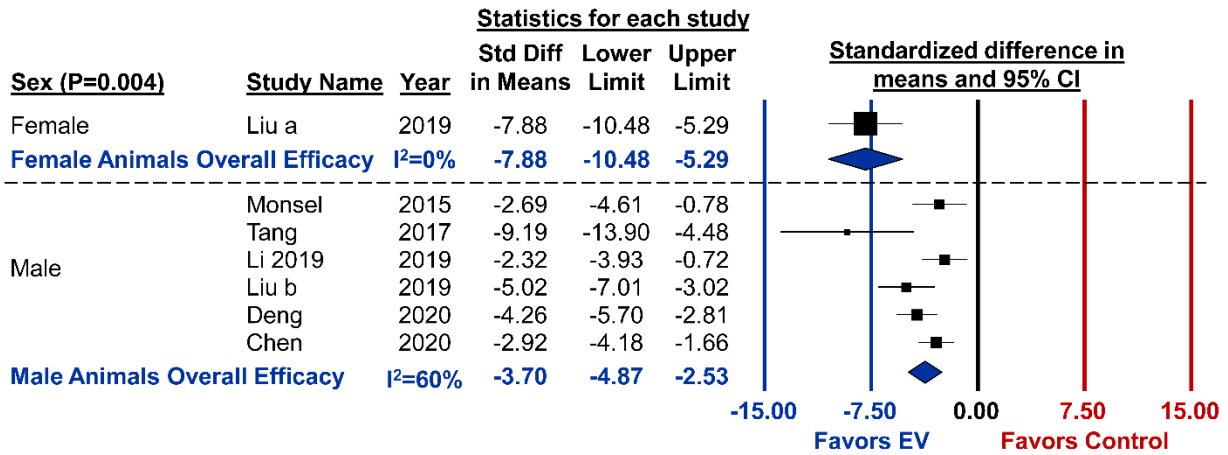


Figure S5.8. Subgroup analysis by sex of treated animals for studies of acute lung injury (ALI) that reported the outcome of lung injury score. Data is presented as a forest plot with standardized mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I^2 value represents the statistical heterogeneity. $P<0.05$ represents a significant difference in EV efficacy between subgroup categories.

Subgroup Analysis of Acute Lung Injury – Alveolar Capillary Permeability:

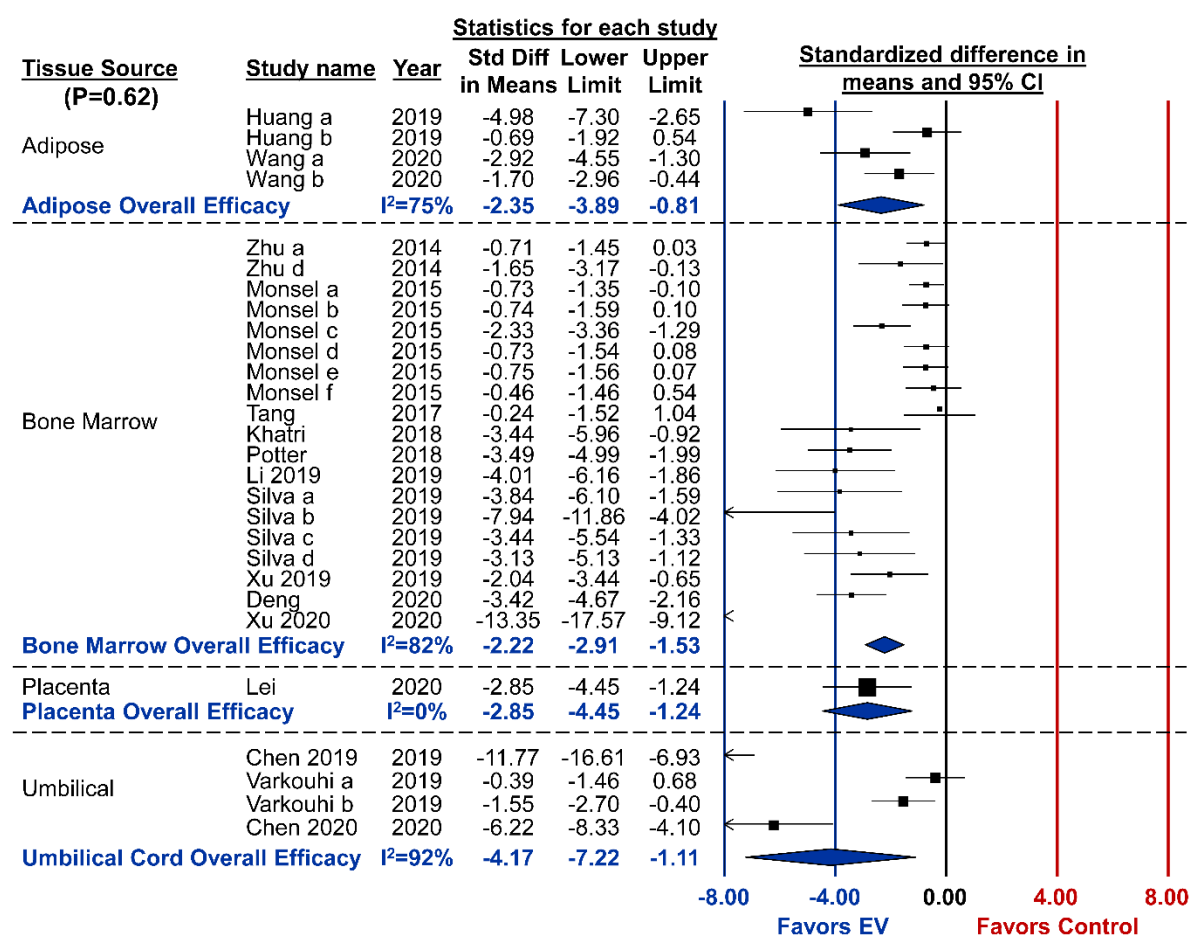


Figure S5.9. Subgroup analysis by MSC tissue source for studies of acute lung injury (ALI) that reported the outcome of alveolar capillary permeability. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity.

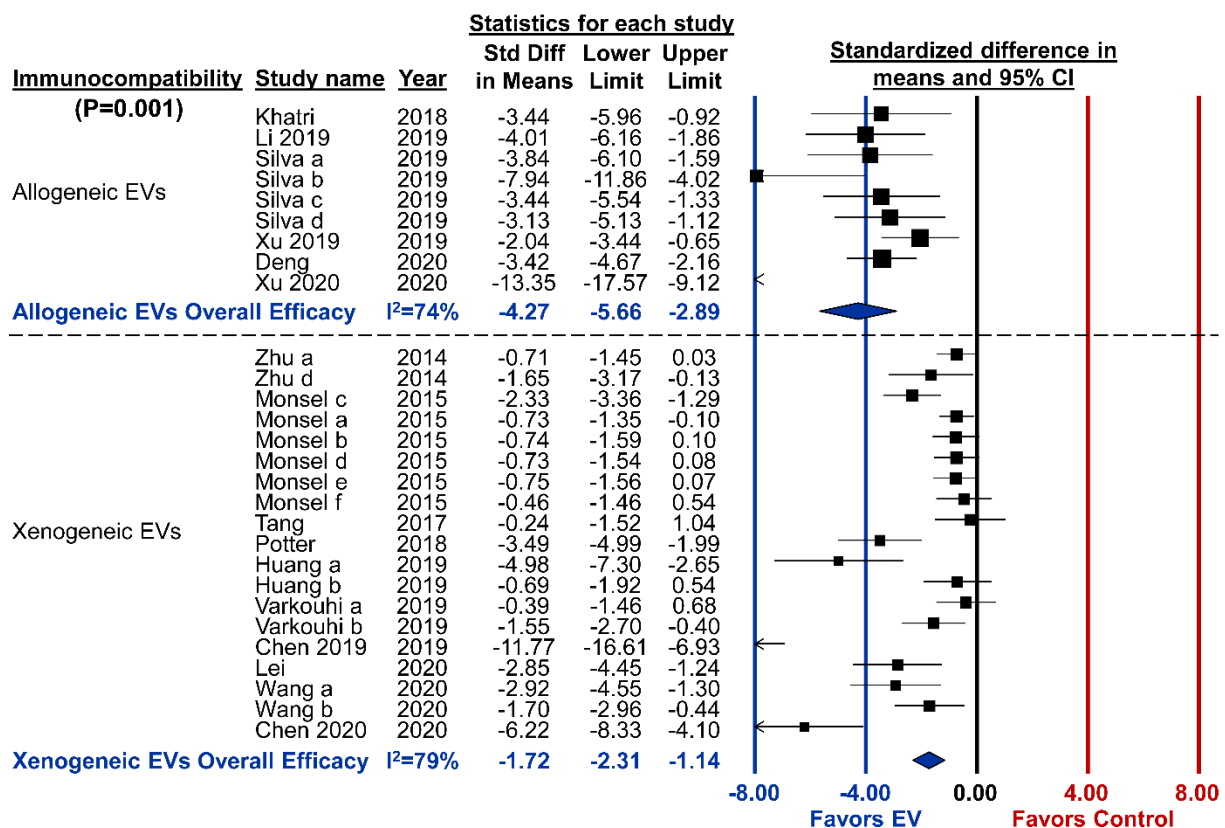


Figure S5.10. Subgroup analysis by EV immunocompatibility for studies of acute lung injury (ALI) that reported the outcome of alveolar capillary permeability. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I^2 value represents the statistical heterogeneity. $P<0.05$ represents a significant difference in EV efficacy between subgroup categories.

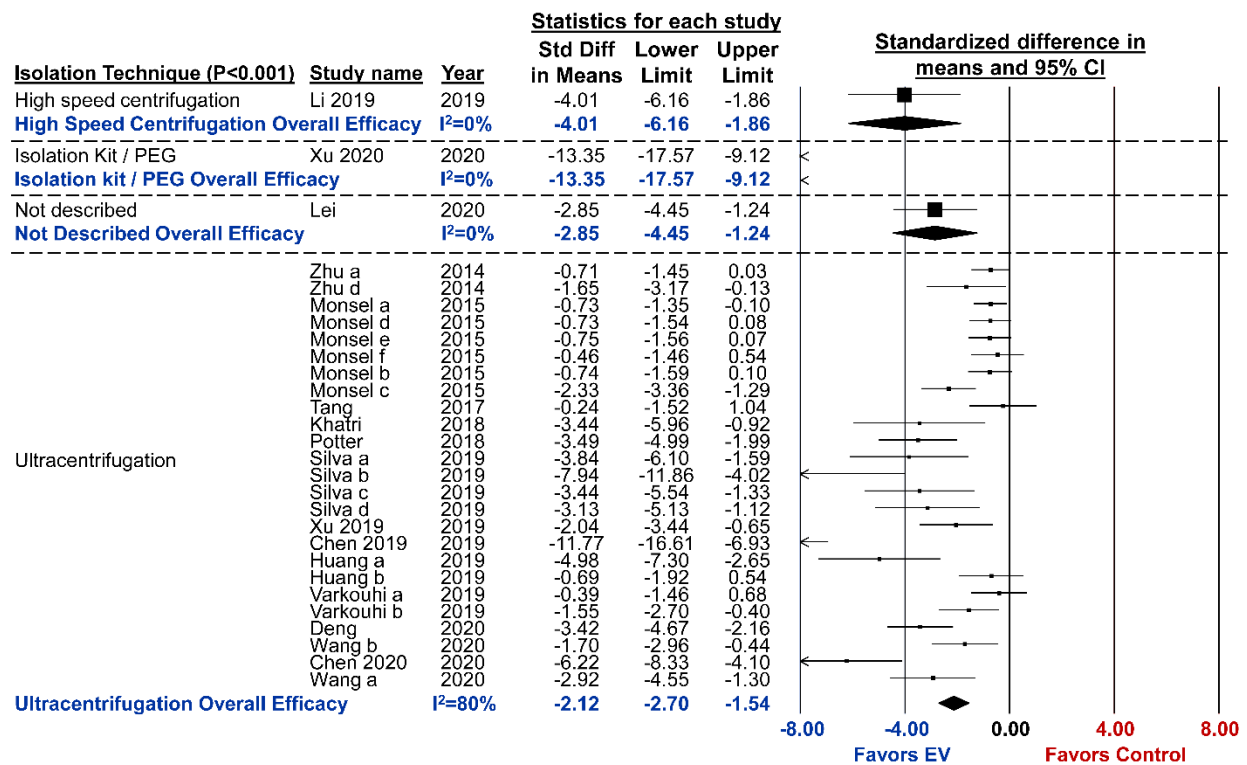


Figure S5.11. Subgroup analysis by EV isolation technique for studies of acute lung injury (ALI) that reported the outcome of alveolar capillary permeability. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity. P<0.05 represents a significant difference in EV efficacy between subgroup categories.

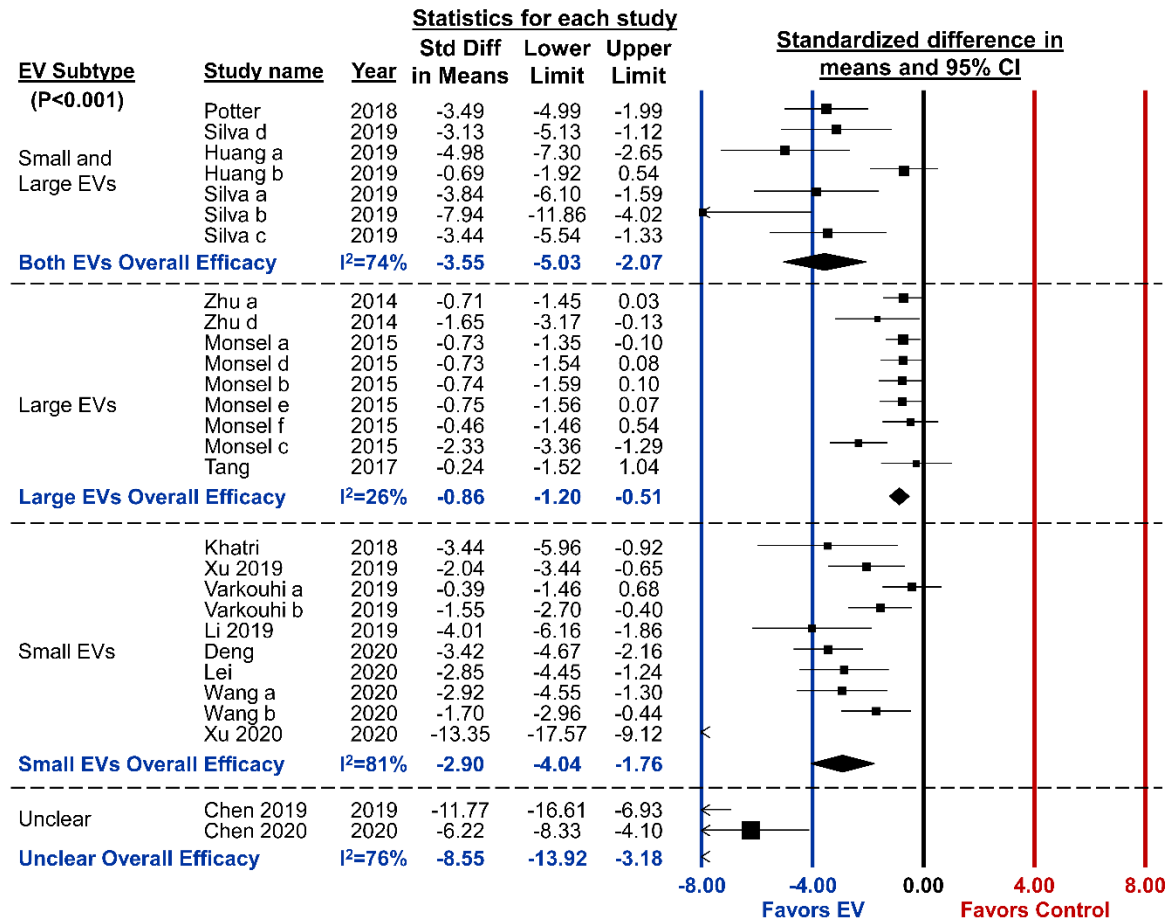


Figure S5.12. Subgroup analysis by EV subtype for studies of acute lung injury (ALI) that reported the outcome of alveolar capillary permeability. Data is presented as a forest plot with standardized mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity. P<0.05 represents a significant difference in EV efficacy between subgroup categories.

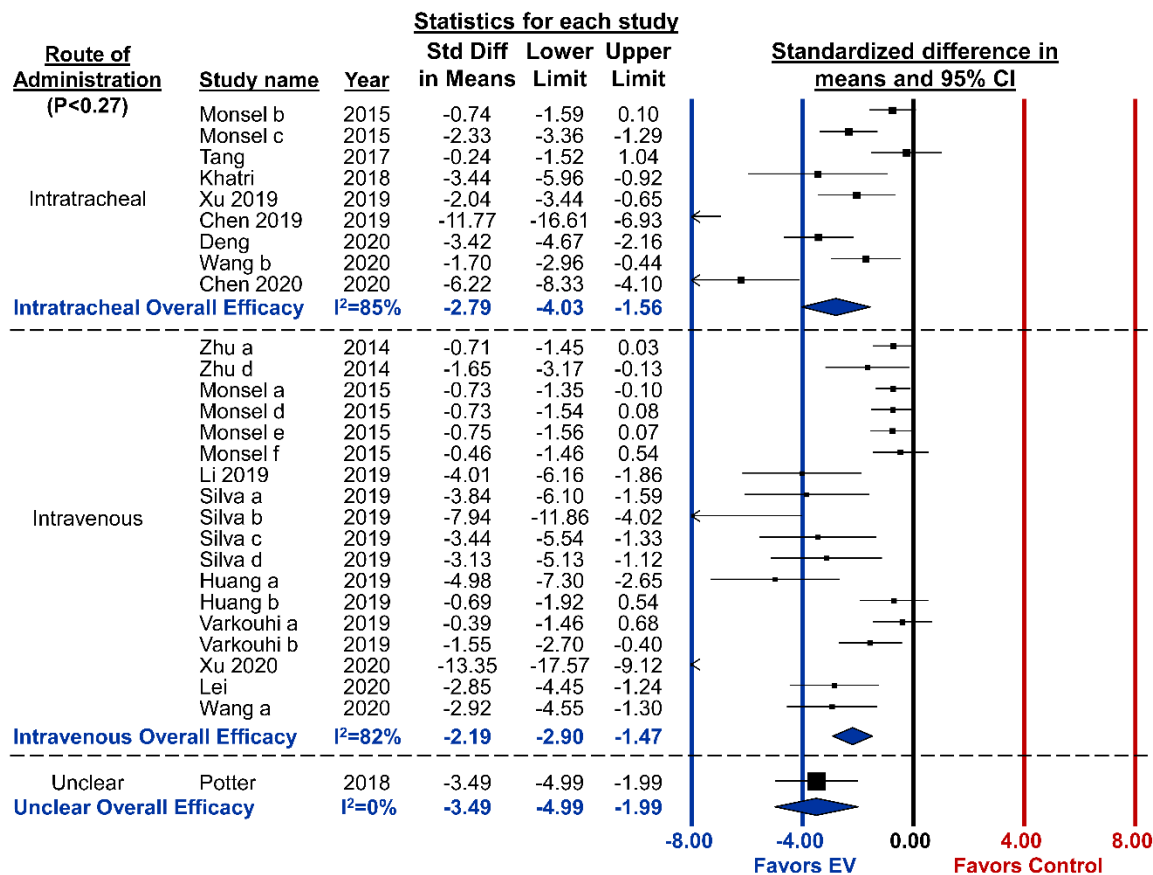


Figure S5.13. Subgroup analysis by route of administration for studies of acute lung injury (ALI) that reported the outcome of alveolar capillary permeability. Data is presented as a forest plot with standardized mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity.

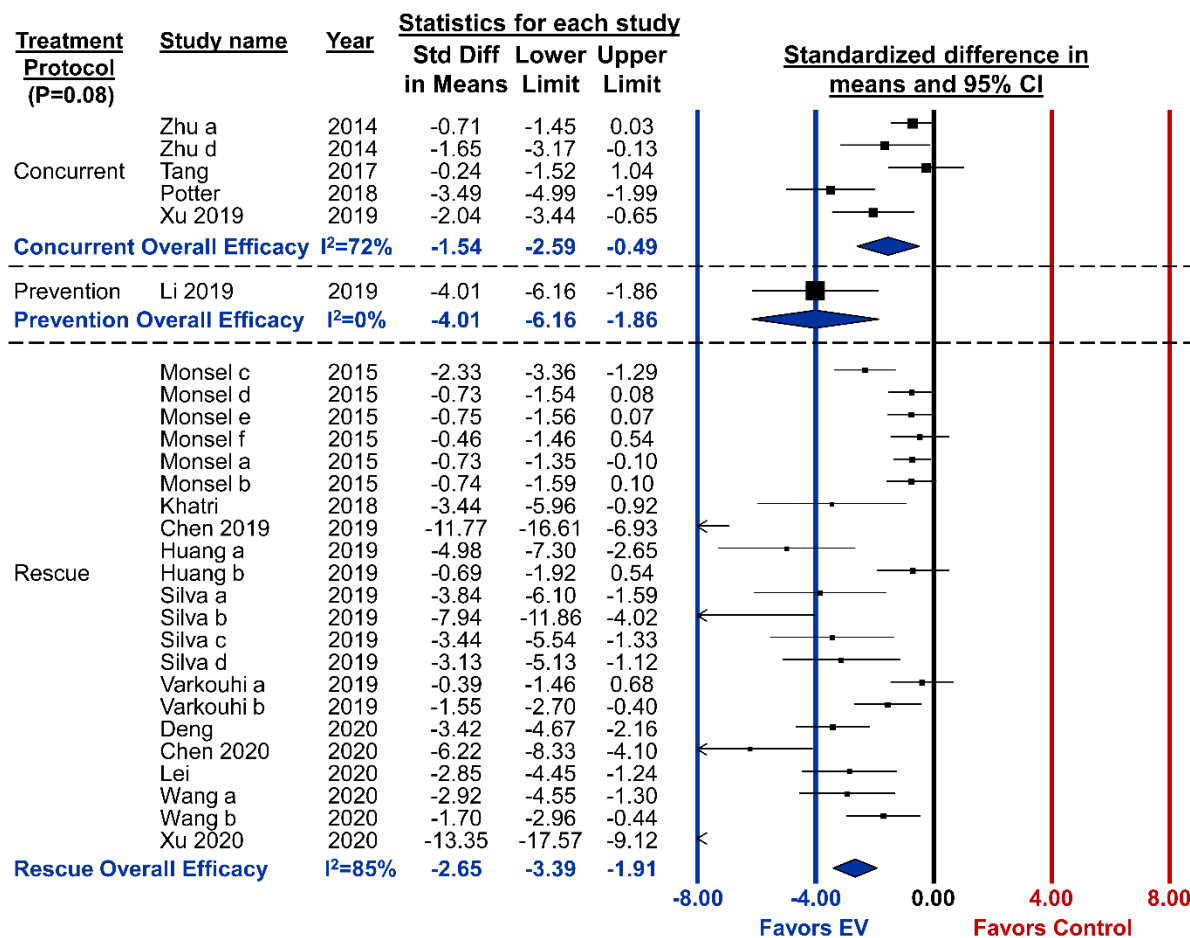


Figure S5.14. Subgroup analysis by timing of treatment for studies of acute lung injury (ALI) that reported the outcome of alveolar capillary permeability. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I^2 value represents the statistical heterogeneity.

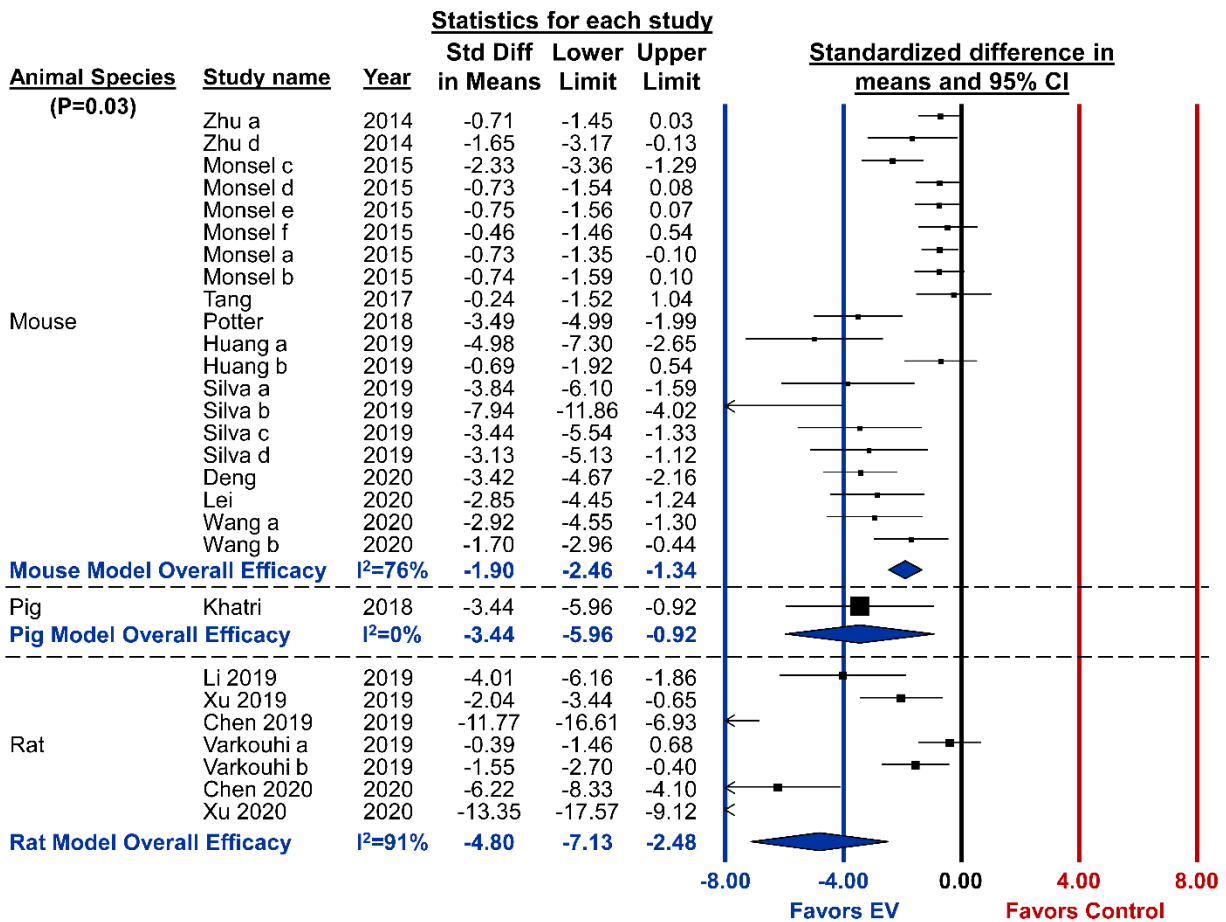


Figure S5.15. Subgroup analysis by species of animal model for studies of acute lung injury (ALI) that reported the outcome of alveolar capillary permeability. Data is presented as a forest plot with standardized mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I^2 value represents the statistical heterogeneity. $P<0.05$ represents a significant difference in EV efficacy between subgroup categories.

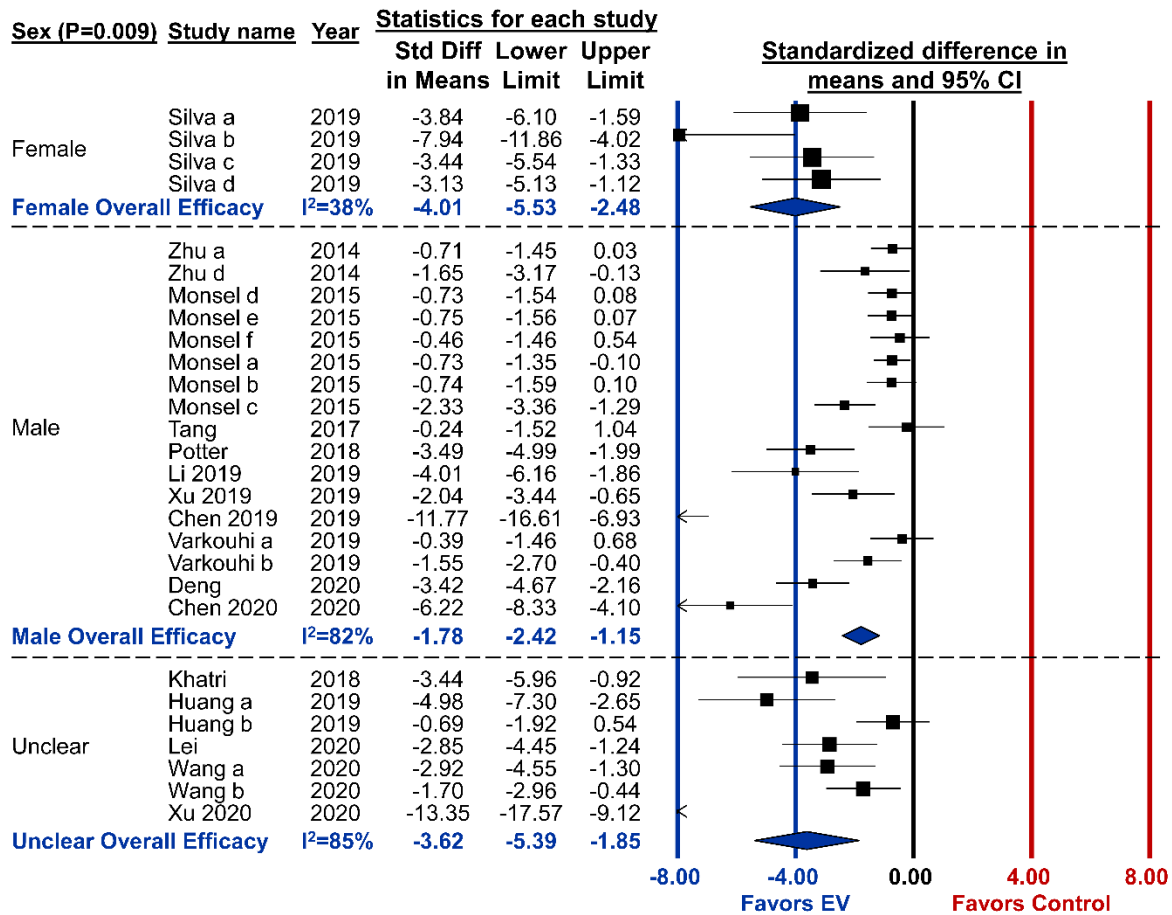


Figure S5.16. Subgroup analysis by sex of treated animals for studies of acute lung injury (ALI) that reported the outcome of alveolar capillary permeability. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity. P<0.05 represents a significant difference in EV efficacy between subgroup categories.

Subgroup Analysis of Bronchopulmonary Dysplasia – Alveolarization:

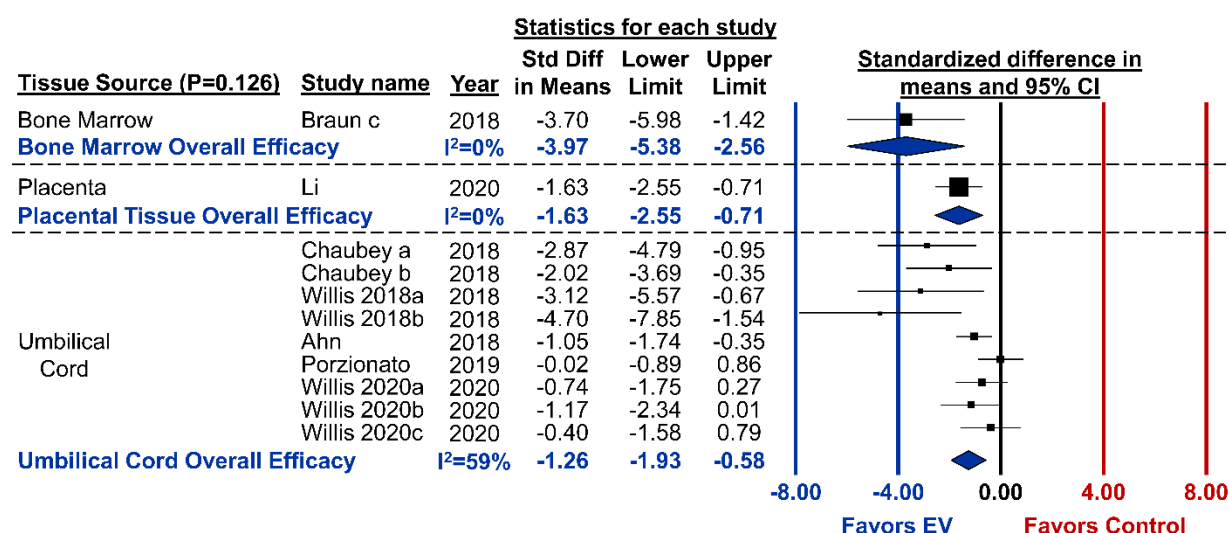


Figure S5.17. Subgroup analysis by MSC tissue source for studies of bronchopulmonary dysplasia (BPD) that reported the outcome of alveolarization. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity.

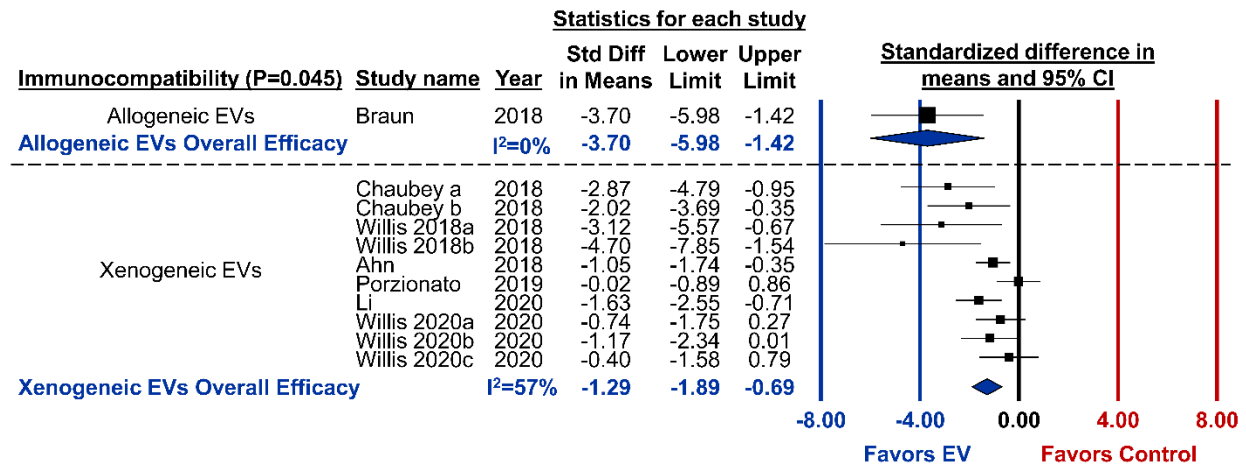


Figure S5.18. Subgroup analysis by EV immunocompatibility for studies of bronchopulmonary dysplasia (BPD) that reported the outcome of alveolarization. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I^2 value represents the statistical heterogeneity. $P<0.05$ represents a significant difference in EV efficacy between subgroup categories.

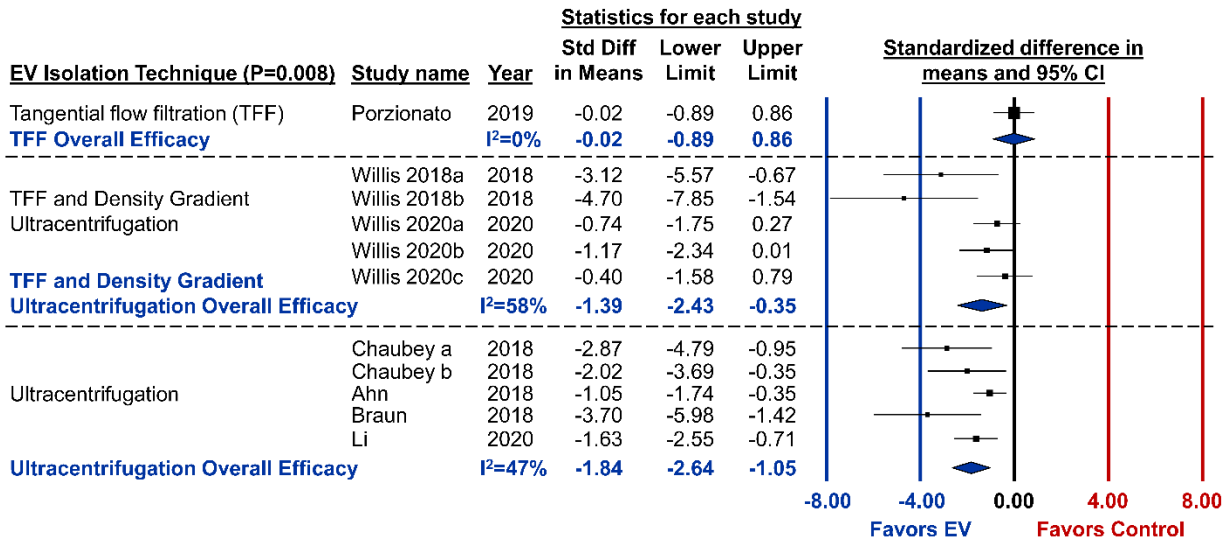


Figure S5.19. Subgroup analysis by EV isolation technique for studies of bronchopulmonary dysplasia (BPD) that reported the outcome of alveolarization. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity. P<0.05 represents a significant difference in EV efficacy between subgroup categories.

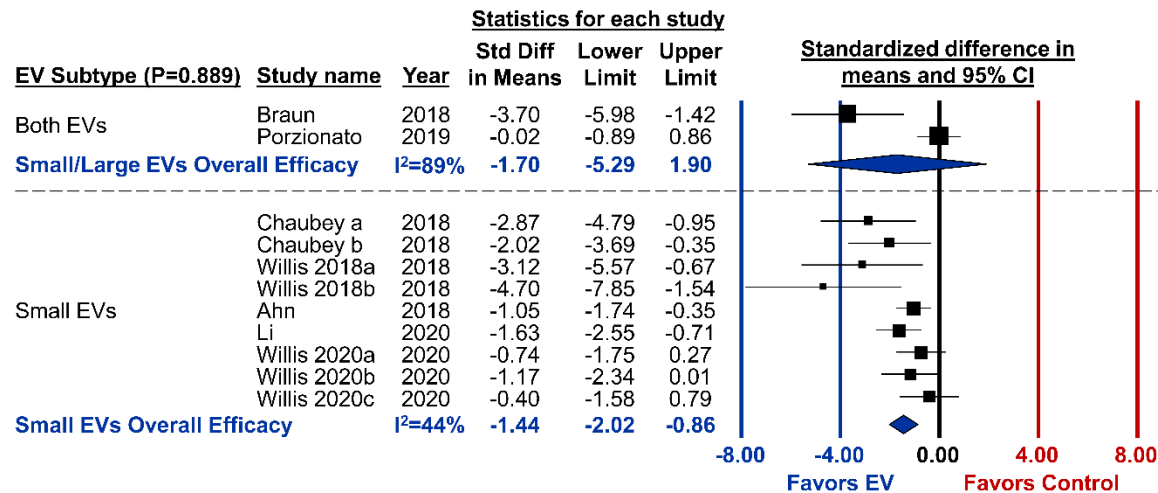


Figure S5.20. Subgroup analysis by EV subtype for studies of bronchopulmonary dysplasia (BPD) that reported the outcome of alveolarization. Data is presented as a forest plot with standardized mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I^2 value represents the statistical heterogeneity.

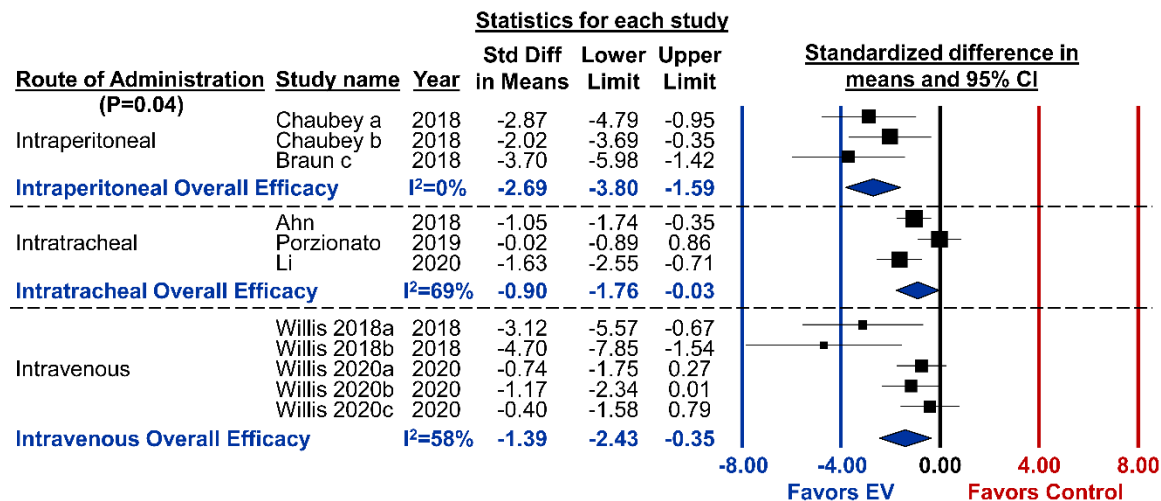


Figure S5.21. Subgroup analysis by route of administration for studies of bronchopulmonary dysplasia (BPD) that reported the outcome of alveolarization. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity. P<0.05 represents a significant difference in EV efficacy between subgroup categories.

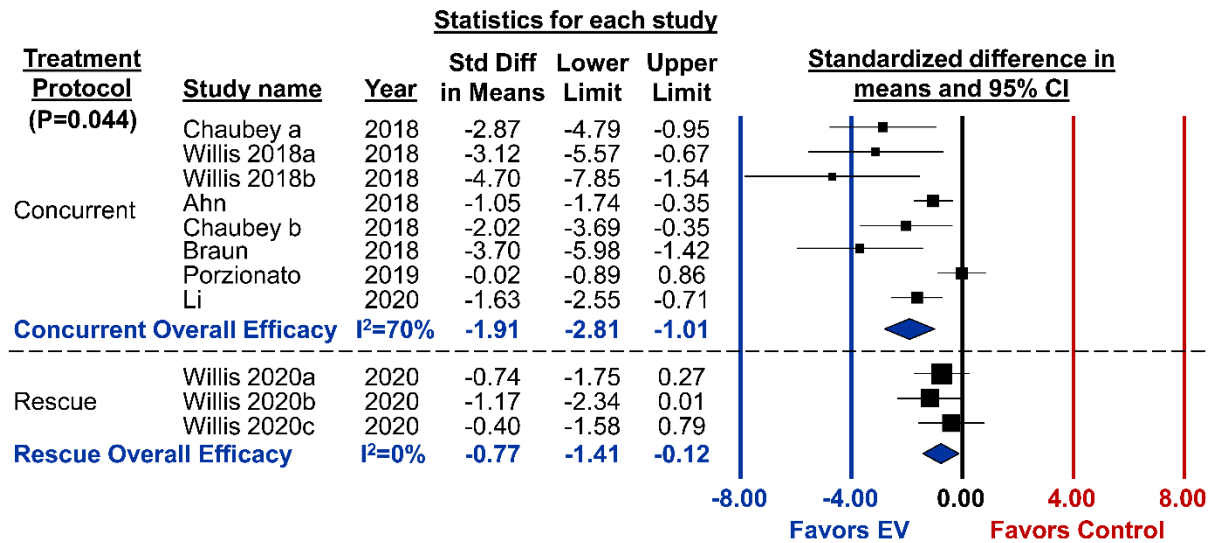


Figure S5.22. Subgroup analysis by timing of treatment for studies of bronchopulmonary dysplasia (BPD) that reported the outcome of alveolarization. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I^2 value represents the statistical heterogeneity. $P<0.05$ represents a significant difference in EV efficacy between subgroup categories.

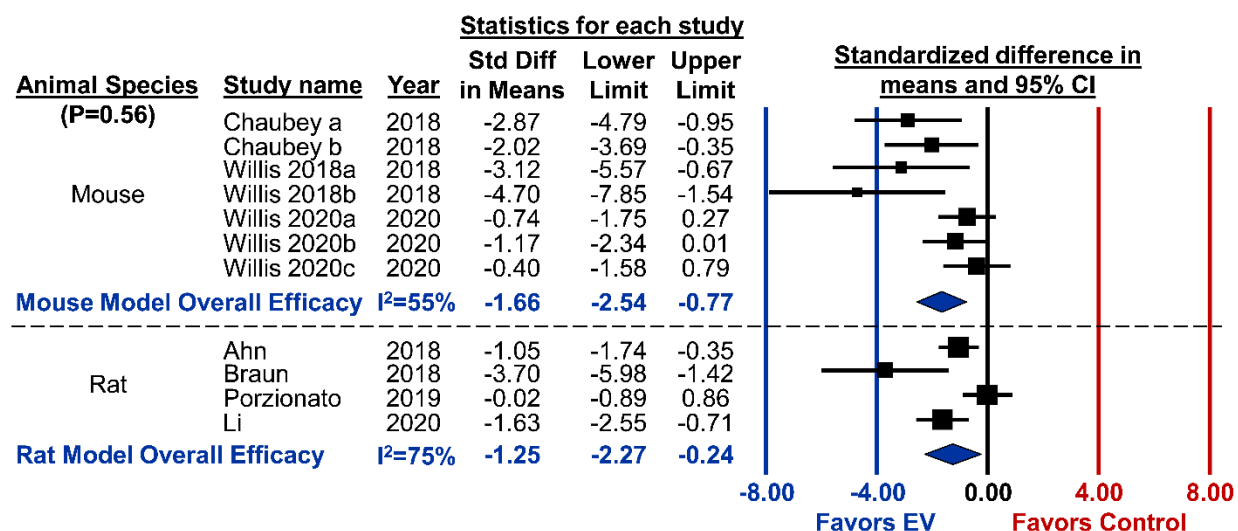


Figure S5.23. Subgroup analysis by species of animal model for studies of bronchopulmonary dysplasia (BPD) that reported the outcome of alveolarization. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I^2 value represents the statistical heterogeneity.

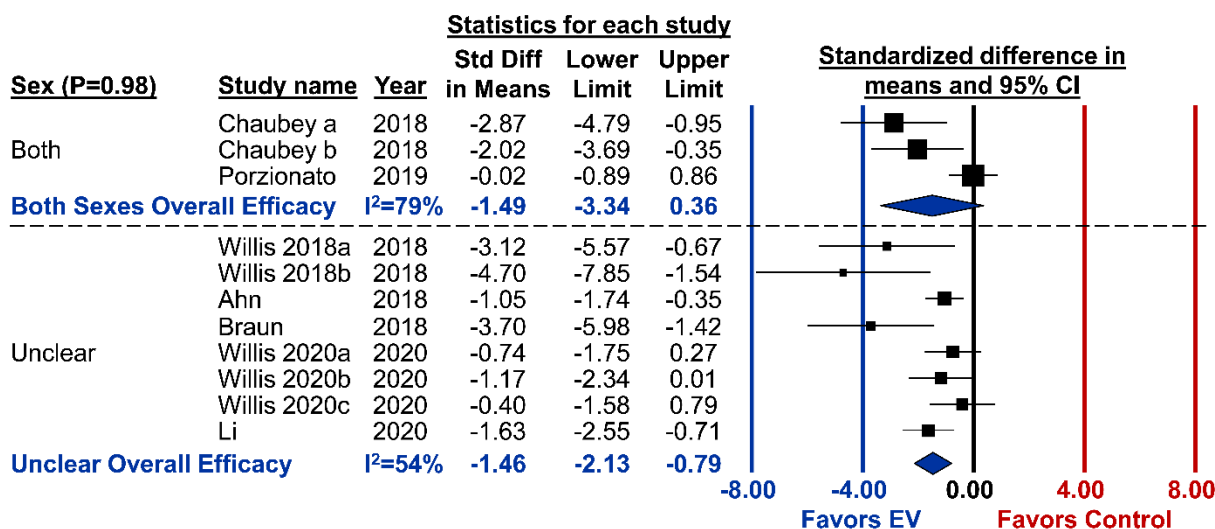


Figure S5.24. Subgroup analysis by sex of treated animals for studies of bronchopulmonary dysplasia (BPD) that reported the outcome of alveolarization. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I^2 value represents the statistical heterogeneity.

Subgroup Analysis of Pulmonary Arterial Hypertension – Right Ventricular Systolic Pressure:

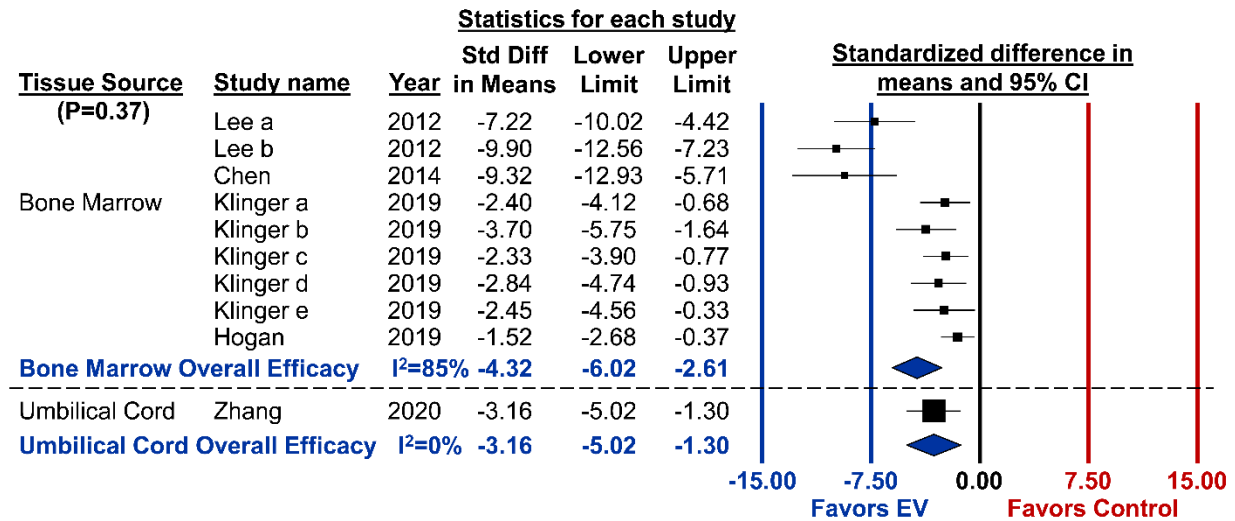


Figure S5.25. Subgroup analysis by MSC tissue source for studies of pulmonary arterial hypertension (PAH) that reported the outcome of right ventricular systolic pressure. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity. P<0.05 represents a significant difference in EV efficacy between subgroup categories.

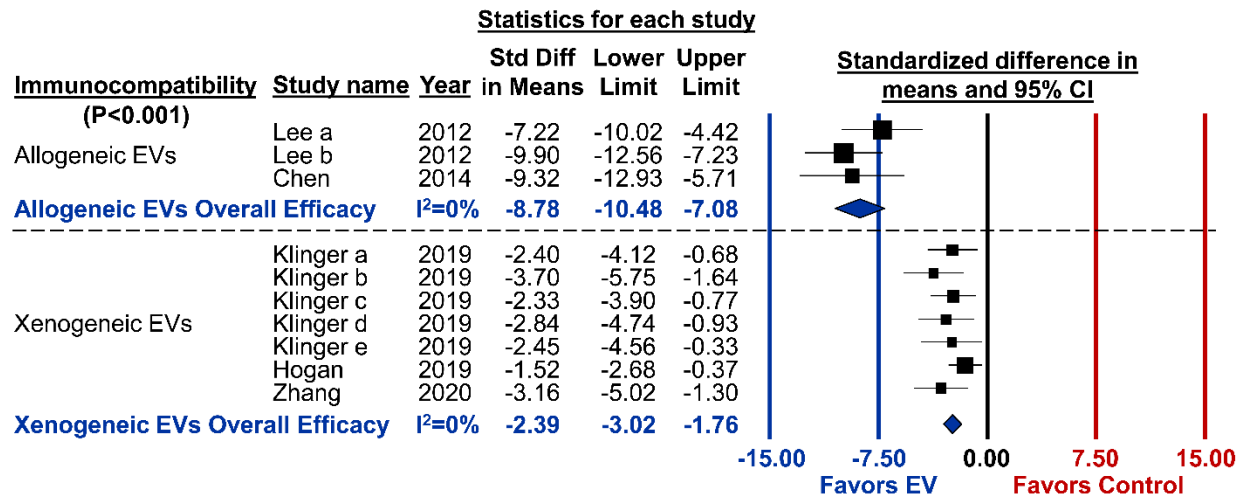


Figure S5.26. Subgroup analysis by EV immunocompatibility for studies of pulmonary arterial hypertension (PAH) that reported the outcome of right ventricular systolic pressure. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity. P<0.05 represents a significant difference in EV efficacy between subgroup categories.

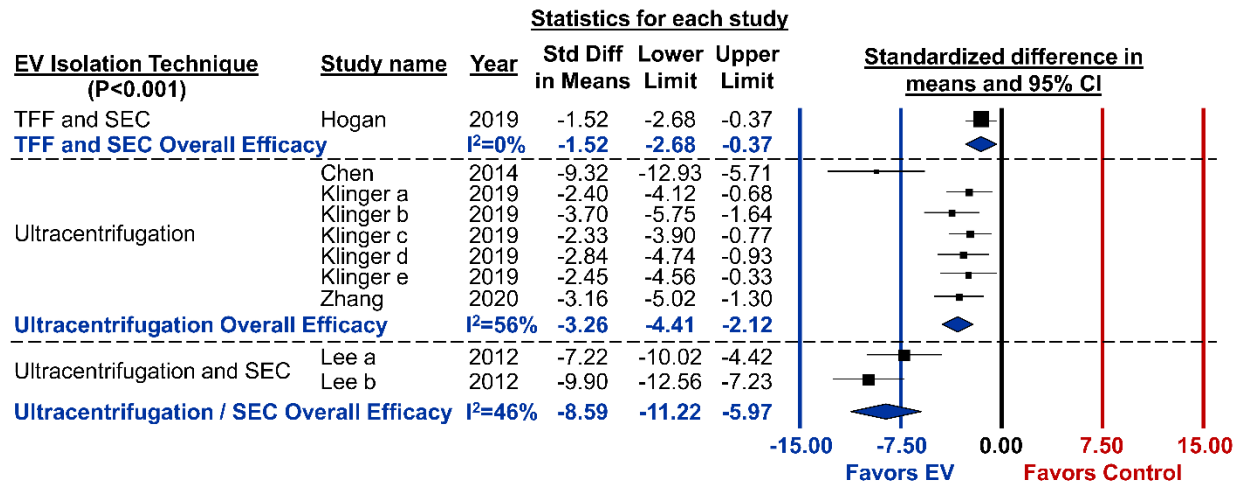


Figure S5.27. Subgroup analysis by EV isolation technique for studies of pulmonary arterial hypertension (PAH) that reported the outcome of right ventricular systolic pressure. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity. P<0.05 represents a significant difference in EV efficacy between subgroup categories.

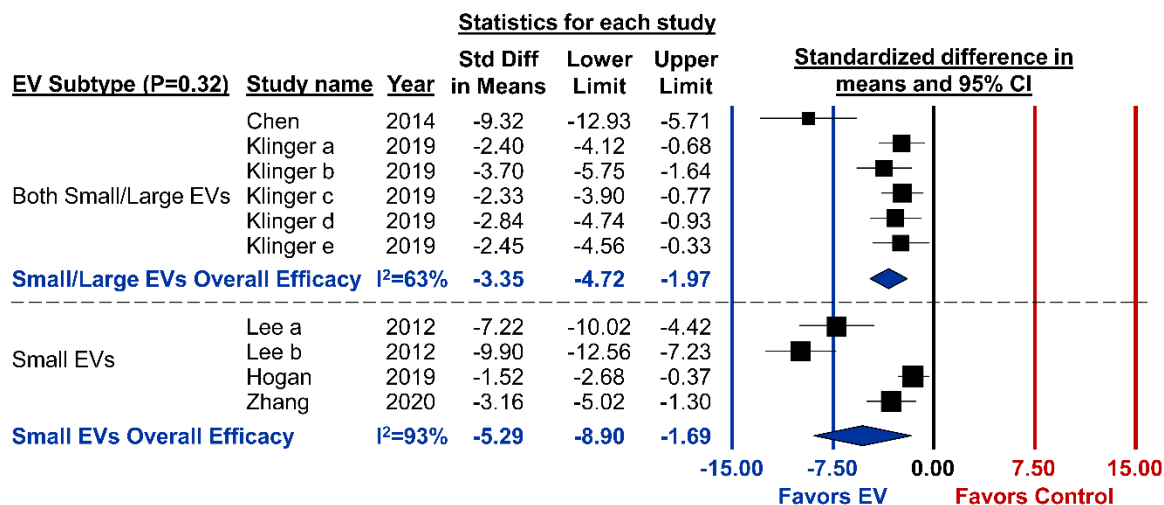


Figure S5.28. Subgroup analysis by EV subtype for studies of pulmonary arterial hypertension (PAH) that reported the outcome of right ventricular systolic pressure. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I^2 value represents the statistical heterogeneity.

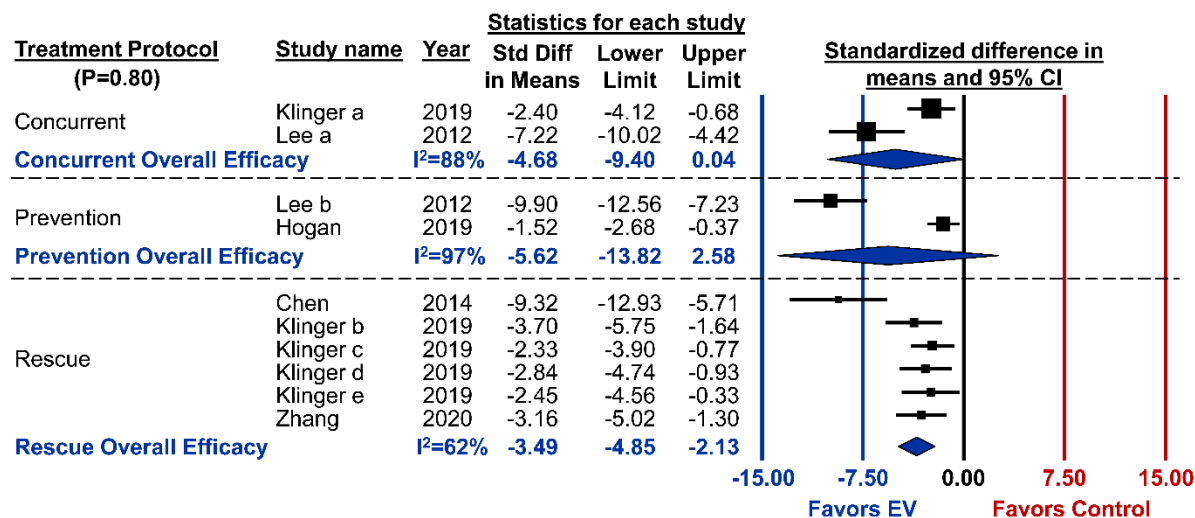


Figure S5.29. Subgroup analysis by timing of treatment for studies of pulmonary arterial hypertension (PAH) that reported the outcome of right ventricular systolic pressure. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity.

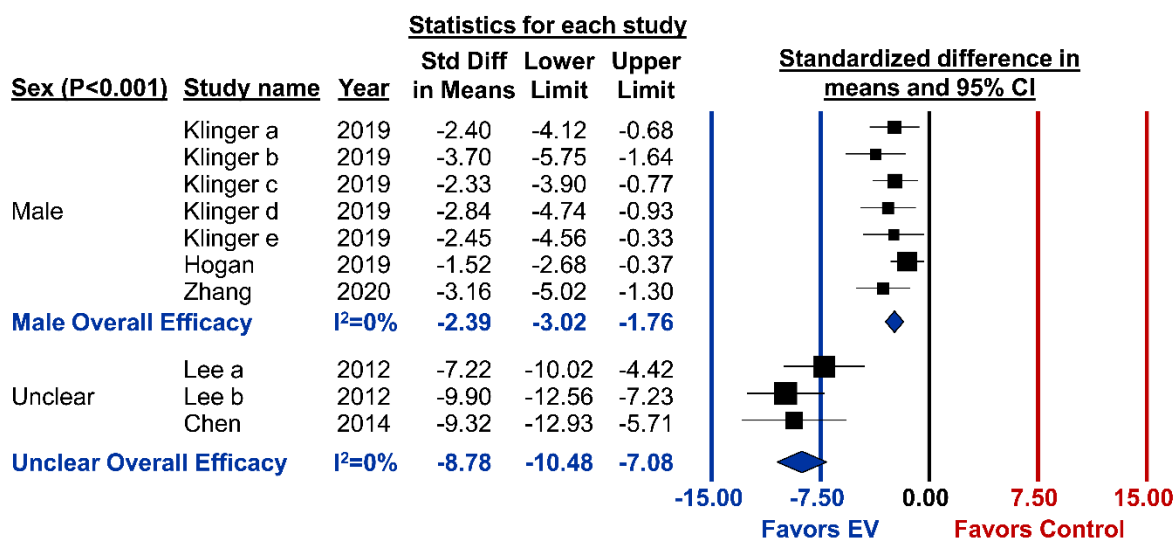


Figure S5.30. Subgroup analysis by sex of treated animals for studies of pulmonary arterial hypertension (PAH) that reported the outcome of right ventricular systolic pressure. Data is presented as a forest plot with standardize mean differences and 95% confidence intervals. Effect sizes <0 favors EV treatment and >0 favors control. Subscript denotes a separate study or article that was published within the same year. The ‘Overall Efficacy’ represents a pooled estimate of MSC-EV effect from all studies within a subgroup. I² value represents the statistical heterogeneity. P<0.05 represents a significant difference in EV efficacy between subgroup categories.

Supplemental File C6 – Data Extraction Form for Study and Intervention Characteristics

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Form Preview

Species of treated animals?

1.

- ☐ Mouse
- ☐ Rat
- ☐ Pig
- ☐ Dog
- ☐ Rabbit
- ☐ Other (add below)

[Permanently add an answer to this question](#)

2. Strain of treated animals?

- ☐ C57/Bl6
- ☐ FVB
- ☐ Sprague Dawley
- ☐ White-Duroc
- ☐ Not Described
- ☐ BL/6J
- ☐ BALB/c
- ☐ CD1
- ☐ mt-Keima
- ☐ A/J Mice

[Permanently add an answer to this question](#)

3. Sex of treated animals?

- ☐ Male only
- ☐ Female only
- ☐ Both
- ☐ Not described

4. Age of animals?

- ☐ Newborn
- ☐ Post natal 4
- ☐ 2-8weeks
- ☐ 5 weeks
- ☐ 6-8 weeks
- ☐ 8 weeks
- ☐ 9-11 wks

<https://v2dis-prod.evidencepartners.com/Submit/RenderForm.php?id=4>

1/13

1/21/23, 4:00 PM

Form Preview

- ☐ 8-12weeks
- ☐ 10-12 weeks
- ☐ 19-21 weeks
- ☐ Not Described
- ☐ 8-10weeks
- ☐ 3-4 weeks
- ☐ 6 weeks
- ☐ 4-6 weeks
- ☐ 4-7 months
- ☐ 14 weeks
- ☐ 4 month
- ☐ 16 weeks
- ☐ 16-28 weeks

Permanently add an answer to this question

5. Human disease modelled?

- ☐ ARDS
- ☐ PAH
- ☐ BPD
- ☐ Asthma / Allergic Airway Inflammation
- ☐ Pneumonia
- ☐ Pulmonary fibrosis
- ☐ Silicosis
- ☐ Ischemia-reperfusion injury
- ☐ CDH
- ☐ Smoke Inhalation Lung Injury
- ☐ Radiation-Induced Lung Injury
- ☐ COPD
- ☐ Burn-induced acute lung injury

Permanently add an answer to this question

6. Model of disease/injury?

- ☐ Hyperoxia (75% O₂)
- ☐ Monocrotaline (MCT) induced
- ☐ Intratracheal Endotoxin
- ☐ Influenza (e.g. H1N1)

<https://v2dis-prod.evidencepartners.com/Submit/RenderForm.php?id=4>

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- ☐ Shock-induced ALI
- ☐ Ischemia-reperfusion
- ☐ Ovalbumin-induced asthma
- ☐ Intratracheal aspergillus hyphal extract (for allergy/asthma)
- ☐ Silica-induced fibrosis
- ☐ Hypoxia (e.g. Mouse PAH)
- ☐ Hyperoxia (85% O2)
- ☐ Hyperoxia (95%) for 4 days
- ☐ Semaxanib (SUGEN)/Chronic Hypoxia (Rat PAH)
- ☐ Hyperoxia (60% O2)
- ☐ Intratracheal E. Coli (pneumonia)
- ☐ Hyperoxia (90% O2)
- ☐ Hilar ligation
- ☐ Infusion of BM cells from MCT-injured mice
- ☐ Oropharyngeal Aspergillus hyphae extract
- ☐ Nitrofen
- ☐ Smoke Chamber (smoldering wood sawdust)
- ☐ Hyperoxia (80% O2)
- ☐ Irradiation dose of 15 Gy
- ☐ Sugen-5416 Injection (VEGF receptor 2 antagonist)
- ☐ PM2.5 (e.g. aerodynamic particles)
- ☐ Cigarette Smoke Exposure
- ☐ Bleomycin injection
- ☐ LPS administered to Lungs
- ☐ Phosgene
- ☐ Rats placed in hot water
- ☐ IL-33 induced allergic inflammation
- ☐ Chests struck by a stainless hammer
- ☐ Burn induced
- ☐ Intestinal ischemia reperfusion
- ☐ Intraperitoneal E Coli
- ☐ Intraperitoneal Endotoxin (LPS)

Permanently add an answer to this question

7. Co-morbidities of treated animals?

- ☐ Not described
- ☐ Intestinal ischemia reperfusion injury

Permanently add an answer to this question

8. Animal source of MSCs?

- ☐ Human
- ☐ Mouse
- ☐ Rat
- ☐ Pig
- ☐ Not described
- ☐ Other (add below)

Permanently add an answer to this question

9. Tissue source of MSCs?

- ☐ Bone marrow
- ☐ Umbilical cord
- ☐ Adipose tissue
- ☐ Menstrual blood
- ☐ Placenta
- ☐ iPSCs
- ☐ Embryonic stem cells
- ☐ Not described
- ☐ Other (add below)

Permanently add an answer to this question

10. Method of MSC characterization?

- ☐ By adherence to plastic dish
- ☐ By differentiation potential

11. Which cell lineages were observed?

- ☐ Osteoblasts
- ☐ Adipocytes
- ☐ Chondroblasts
- ☐ Other (add below)
- ☐ Not described

Permanently add an answer to this question

☐ By surface markers

13. Method used?

- ☐ Western blot
- ☐ Flow cytometry
- ☐ ELISA
- ☐ Not described

[Permanently add an answer to this question](#)

14. Positive markers detected? (> 95%)

- ☐ CD105
- ☐ CD73
- ☐ CD90
- ☐ CD44
- ☐ CD29 (Integrin beta1)
- ☐ Not described
- ☐ Sca1+
- ☐ c-kit
- ☐ Calnexin
- ☐ GM130
- ☐ CD63
- ☐ CD166

[Permanently add an answer to this question](#)

15. Negative markers detected? (<= 2%)

- ☐ CD45
- ☐ CD34
- ☐ CD14 or CD11b
- ☐ CD79alpha or CD19
- ☐ HLA/DR
- ☐ CD31
- ☐ Not described
- ☐ CD86
- ☐ CD(
- ☐ CD9
- ☐ CD63
- ☐ CD81

- ☐ Alix
- ☐ TSG101
- ☐ CD117

Permanently add an answer to this question

- ☐ Not Described

Permanently add an answer to this question

16. Method of EV isolation?

- ☐ Ultracentrifugation
- ☐ Isolation kit / PEG (Polyethylene Glycol precipitation)
- ☐ Tangential flow filtration
- ☐ Size exclusion chromatography
- ☐ Sucrose density gradient centrifugation
- ☐ Ultrafiltration
- ☐ High speed centrifugation
- ☐ Other (add below)
- ☐ IDX Density gradient ultracentrifugation
- ☐ Not described
- ☐ Sequential filtration
- ☐ μ MACS Separator
- ☐ Anion-exchange chromatography

Permanently add an answer to this question

17. Method of EV characterization?

- ☐ By size

18. Method used?

- ☐ Nanoparticle tracking analysis
- ☐ Dynamic light scattering
- ☐ Tunable resistive pulse sensing
- ☐ Scanning EM
- ☐ TEM
- ☐ Not Described

Permanently add an answer to this question

☐ By surface markers

19. Method used?

- ☐ Western blot
- ☐ Flow cytometry
- ☐ ELISA

[Permanently add an answer to this question](#)

20. Positive markers detected?

- ☐ CD63
- ☐ CD9
- ☐ CD81
- ☐ TSG101
- ☐ Alix
- ☐ HSP70/90
- ☐ Flotillin-1
- ☐ Other (add below)
- ☐ CD29 (Integrin B1/B2)
- ☐ Annexin V
- ☐ MSC markers
- ☐ Lamp1
- ☐ EpCAM
- ☐ ICAM
- ☐ CD44
- ☐ TGN48
- ☐ CD105
- ☐ CD40
- ☐ PDCC6IP
- ☐ GAPDH
- ☐ Not Described
- ☐ cd90

[Permanently add an answer to this question](#)

21. Negative markers detected?

- ☐ Calnexin
- ☐ Cytochrome C
- ☐ GM130

1/21/23, 4:00 PM

Form Preview

- ☐ Not Described
- ☐ Other (add below)
- ☐ Fibrillarin
- ☐ HLAs
- ☐ TGN48
- ☐ NFkB1
- ☐ albumin
- ☐ CD44
- ☐ CD146
- ☐ CD73
- ☐ CD90
- ☐ CD105

Permanently add an answer to this question

- ☐ By single vesicle analysis (e.g. electron microscopy)

22. Method used?

- ☐ Transmission Electron microscopy
- ☐ Scanning Electron Microscopy
- ☐ Atomic Force Microscopy
- ☐ Not Described
- ☐ Other (add below)

Permanently add an answer to this question

☐ By amount (protein or particle)

23. Method used?

- ☐ NTA
- ☐ Bradford Assay
- ☐ BCA (Bicinchoninic Acid) Assay
- ☐ DC / Lowry Assay
- ☐ UV Absorption at 280nm (e.g. spectrophotometer)
- ☐ Not Described
- ☐ Other (add below)
- ☐ NanoDrop (for RNA concentration)
- ☐ Qubit Fluorometer
- ☐ Qubit RNA BR assay kit
- ☐ Tunable resistive pulse sensing (e.g. Izon QNano)
- ☐ Flow Cytometry
- ☐ Bio-Rad Protein Assay

[Permanently add an answer to this question](#)

☐ Other (add below)

☐ Not Described

[Permanently add an answer to this question](#)

24. Size of isolated EVs?

- ☐ 30 - 150 nm (exosomes)
- ☐ 150 - 1000 nm (MVs)
- ☐ 30 - 1000 nm (both used together)
- ☐ Both (used in separate experiments)
- ☐ Not described

[Permanently add an answer to this question](#)

25. Terminology used to describe isolated EVs?

- ☐ Exosomes
- ☐ MVs
- ☐ EVs
- ☐ Small EVs
- ☐ Large EVs
- ☐ Microparticles
- ☐ Other (add below)

Permanently add an answer to this question

26. Consistency between characteristics of isolated EVs (e.g. size) and terminology used to describe them?

- ☐ Yes
☐ No
☐ Unclear

Clear Response

27. Method of EV storage?

- ☐ Fresh/Fridge (4C)
☐ Stored at -20C
☐ Frozen at -80°C
☐ Liquid Nitrogen (Cryopreserved)
☐ Not described
☐ Other (add below)
☐ Frozen at -70C

Permanently add an answer to this question

28. Immunocompatibility of EVs?

- ☐ Xenogeneic
☐ Allogeneic
☐ Syngeneic
☐ Autologous
☐ Not Described

29. Were EVs Modified?

- ☐ Unmodified
☐ Modified Before EV Isolation (e.g. MSCs were modified)
☐ Modified After EV Isolation (e.g. EVs directly)
☐ Other (add answer below)
☐ Co-treated with ACE2 inhibitor (A-779)

Permanently add an answer to this question

30. Co-administration of EVs with another treatment (experimental group 1)?

- ☐ Antibiotics
☐ Fluids
☐ Ventilation
☐ None
☐ Other (add below)

- ☐ A-779 (inhibitor of ACE2 receptor)
- ☐ Sildenafil
- ☐ Anti-KGF antibody
- ☐ Anti-CD44 Antibody
- ☐ TSG-6 nAb
- ☐ LY293111 (LTB4 inhibitor)
- ☐ P2X7 vector
- ☐ MSCs

Permanently add an answer to this question

31. Route of EV administration?

- ☐ Intravenous
- ☐ Intra-arterial
- ☐ Intratracheal
- ☐ Direct tissue injection
- ☐ Subcutaneous
- ☐ Not Described
- ☐ intraperitoneal
- ☐ Intravascular
- ☐ Intracardiac
- ☐ Intranasal
- ☐ Inhjalation
- ☐ Inhalation

Permanently add an answer to this question

32. Units of EV dose?

- ☐ EV protein amount
- ☐ EV particle number
- ☐ EV protein amount / weight of animal
- ☐ EV particle number / weight of animal
- ☐ Dose based on EVs released by a certain number of MSCs
- ☐ Not Described
- ☐ Other (add below)
- ☐ EVs made over time by certain number of MSCs
- ☐ Unclear
- ☐ RNA Concentration

Permanently add an answer to this question

33. Specific EV dose?

34. Timing of first EV dose administration?

- ☐ Prior to injury/disease induction
- ☐ After injury/disease induction
- ☐ At time of injury/disease induction

35. Number of EV doses?

- ☐ Single dose
- ☐ Multiple doses

36. If multiple doses, how many doses were given to each animal in total?

- ☐ 1
- ☐ 2
- ☐ 3
- ☐ 4
- ☐ 5
- ☐ 7
- ☐ 15
- ☐ 8
- ☐ 9
- ☐ 14
- ☐ 12
- ☐ 10

[Permanently add an answer to this question](#)

37. At what timepoints / at which frequency?

38. Was a dose-response study conducted?

- ☐ Yes
- ☐ Not described

[Permanently add an answer to this question](#)

39. Was an in vivo biodistribution experiment completed?

- ☐ Yes
- ☐ No

Permanently add an answer to this question

40. Country of corresponding author?

- ☐ China
- ☐ United States
- ☐ South Korea
- ☐ Other (add below)
- ☐ Brazil
- ☐ Italy
- ☐ Serbia
- ☐ Ireland
- ☐ Canada

Permanently add an answer to this question

41. Source of funding?

- ☐ Private
- ☐ Academic
- ☐ Both
- ☐ Not described

42. Year of publication?

Supplemental File C7 – Data Extraction Form for Outcomes

RefID: 1. There and Back Again: A Review of Residency and Return Migrations in Sharks, with Implications for Population Structure and Management Chapman DD, Feldheim KA, Papastamatiou Y, Hueter RE

The overexploitation of sharks has become a global environmental issue in need of a comprehensive and multifaceted management response. Tracking studies are beginning to elucidate how shark movements shape the internal dynamics and structure of populations, which determine the most appropriate scale of these management efforts.

Tracked sharks frequently either remain in a restricted geographic area for an extended period of time (residency) or return to a previously resided-in area after making long-distance movements (site fidelity). Genetic studies have shown that some individuals of certain species preferentially return to their exact birthplaces (natal philopatry) or birth regions (regional philopatry) for either parturition or mating, even though they make long-distance movements that would allow them to breed elsewhere. More than 80 peer-reviewed articles, constituting the majority of published shark tracking and population genetic studies, provide evidence of at least one of these behaviors in a combined 31 shark species from six of the eight extant orders.

Residency, site fidelity, and philopatry can alone or in combination structure many coastal shark populations on finer geographic scales than expected based on their potential for dispersal. This information should therefore be used to scale and inform assessment, management, and conservation activities intended to restore depleted shark populations. Expected final online publication date for the Annual Review of Marine Science Volume 7 is January 03, 2015.

1. What were the primary outcomes measured?

☒ Alveolar-capillary membrane permeability (ALI)

2. How was this parameter measured?

☒ Total protein concentration in BALF

3. Units?

--

4. What was the comparator/control group?

☐ Animals treated with vehicle

☐ Animals treated with MSC-conditioned media

☐ Animals treated with MSCs

☐ Animals treated with EVs derived from another cell type (e.g. fibroblasts)

☐ Other (add below)

Permanently add an answer to this question ()

5. Sample size (comparator/control group)

--

6. Mean of outcome (comparator/control group)

--

7. Standard deviation of outcome (comparator/control group)

--

8. How many experimental groups were there (i.e. groups treated with MSC-EVs)?

☐ 1

☐ 2

☐ 3

Permanently add an answer to this question ()

Clear Response ()

9. What was the experimental group 1?

☐ Animals treated with unmodified MSC-EVs

☐ Animals treated with EVs derived from pre-treated MSCs (specify, e.g. hypoxic culture conditions)

☐ Animals treated with EVs derived from modified MSCs (specify, e.g. transfected MSCs)

☐ Animals treated with modified EVs (specify, e.g. transfected EVs)

☐ Animals treated with EVs co-administered with another treatment (specify, e.g. antibiotics)

☐ Other (add below)

Permanently add an answer to this question ()

10. Sample size (experimental group 1)

--

11. Mean of outcome (experimental group 1)

--

12. Standard deviation of outcome (experimental group 1)

--

13. Latest timepoint at which outcome was measured

--

☐ Other (add below)

Permanently add an answer to this question ()

☒ Alveolarization (BPD)

14. How was this parameter measured?

☒ Mean linear intercept

15. Units?

...

16. What was the comparator/control group?

☐ Animals treated with vehicle☐ Animals treated with MSC-conditioned media☐ Animals treated with MSCs☐ Animals treated with EVs derived from another cell type (e.g. fibroblasts)☐ Other (add below)

Permanently add an answer to this question {}

17. Sample size of comparator/control group

...

18. Mean of outcome (comparator/control group)

...

19. Standard deviation of outcome (comparator/control group)

...

20. How many experimental groups were there (i.e. groups treated with MSC-EVs)?

☐ 1☐ 2☐ 3

Permanently add an answer to this question {}

Clear Response {}

21. What was the experimental group 1?

☐ Animals treated with unmodified MSC-EVs☐ Animals treated with EVs derived from pre-treated MSCs (specify, e.g. hypoxic culture conditions)☐ Animals treated with EVs derived from modified MSCs (specify, e.g. transfected MSCs)☐ Animals treated with modified EVs (specify, e.g. transfected EVs)☐ Animals treated with EVs co-administered with another treatment (specify, e.g. antibiotics)☐ Other (add below)

Permanently add an answer to this question {}

22. Sample size (experimental group 1)

...

23. Mean of outcome (experimental group 1)

...

24. Standard deviation of outcome (experimental group 1)

...

25. Latest timepoint at which outcome was measured

...

☐ Other (add below)

Permanently add an answer to this question {}

☒ Pulmonary hypertension (PAH)

26. How was this parameter measured?

☒ RVSP

27. Units?

...

28. What was the comparator/control group?

- ☐ Animals treated with vehicle
- ☐ Animals treated with MSC-conditioned media
- ☐ Animals treated with MSCs
- ☐ Animals treated with EVs derived from another cell type (e.g. fibroblasts)
- ☐ Other (add below)

[Permanently add an answer to this question \(\)](#)

29. Sample size of comparator/control group

...

30. Mean of outcome (comparator/control group)

...

31. Standard deviation of outcome (comparator/control group)

...

32. How many experimental groups were there (i.e. groups treated with MSC-EVs)?

- ☐ 1
- ☐ 2
- ☐ 3

[Permanently add an answer to this question \(\)](#)
[Clear Response \(\)](#)

33. What was the experimental group 1?

- ☐ Animals treated with unmodified MSC-EVs
- ☐ Animals treated with EVs derived from pre-treated MSCs (specify, e.g. hypoxic culture conditions)
- ☐ Animals treated with EVs derived from modified MSCs (specify, e.g. transfected MSCs)
- ☐ Animals treated with modified EVs (specify, e.g. transfected EVs)
- ☐ Animals treated with EVs co-administered with another treatment (specify, e.g. antibiotics)
- ☐ Other (add below)

[Permanently add an answer to this question \(\)](#)

34. Sample size (experimental group 1)

...

35. Mean of outcome (experimental group 1)

...

36. Standard deviation of outcome (experimental group 1)

...

37. Latest timepoint at which outcome was measured

...

Form Preview

☒ Right ventricle hypertrophy (Fulton's index)

49. What was the comparator/control group?

- ☐ Animals treated with vehicle
- ☐ Animals treated with MSC-conditioned media
- ☐ Animals treated with MSCs
- ☐ Animals treated with EVs derived from another cell type (e.g. fibroblasts)
- ☐ Other (add below)

Permanently add an answer to this question {}

50. Sample size of the comparator/control group

...

51. Mean of outcome (comparator/control group)

...

52. Standard deviation of outcome (comparator/control group)

...

53. How many experimental groups were there (i.e. groups treated with MSC-EVs)?

- ☐ 1
- ☐ 2
- ☐ 3

Permanently add an answer to this question {}

Clear Response {}

54. What was the experimental group 1?

- ☐ Animals treated with unmodified MSC-EVs
- ☐ Animals treated with EVs derived from pre-treated MSCs (specify, e.g. hypoxic culture conditions)
- ☐ Animals treated with EVs derived from modified MSCs (specify, e.g. transfected MSCs)
- ☐ Animals treated with modified EVs (specify, e.g. transfected EVs)
- ☐ Animals treated with EVs co-administered with another treatment (specify, e.g. antibiotics)
- ☐ Other (add below)

Permanently add an answer to this question {}

55. Sample size (experimental group 1)

...

56. Mean of outcome (experimental group 1)

...

57. Standard deviation of outcome (experimental group 1)

...

58. Latest timepoint at which outcome was measured

...

☒ mPAP

38. Units?

39. What was the comparator/control group?

- ☐ Animals treated with vehicle
- ☐ Animals treated with MSC-conditioned media
- ☐ Animals treated with MSCs
- ☐ Animals treated with EVs derived from another cell type (e.g. fibroblasts)
- ☐ Other (add below)

Permanently add an answer to this question ()

40. Sample size of comparator/control group

41. Mean of outcome (comparator/control group)

42. Standard deviation of outcome (comparator/control group)

43. How many experimental groups were there (i.e. groups treated with MSC-EVs)?

- ☐ 1
- ☐ 2
- ☐ 3

Permanently add an answer to this question ()

Clear Response ()

44. What was the experimental group 1?

- ☐ Animals treated with unmodified MSC-EVs
- ☐ Animals treated with EVs derived from pre-treated MSCs (specify, e.g. hypoxic culture conditions)
- ☐ Animals treated with EVs derived from modified MSCs (specify, e.g. transfected MSCs)
- ☐ Animals treated with modified EVs (specify, e.g. transfected EVs)
- ☐ Animals treated with EVs co-administered with another treatment (specify, e.g. antibiotics)
- ☐ Other (add below)

Permanently add an answer to this question ()

45. Sample size (experimental group 1)

46. Mean of outcome (experimental group 1)

47. Standard deviation of outcome (experimental group 1)

48. Latest timepoint at which outcome was measured

☐ Other (add below)

Permanently add an answer to this question ()

☐ Other (add below)

Permanently add an answer to this question ()

59. What were the secondary outcomes measured?

☒ Histological lung injury score (ALI)

60. What was the comparator/control group?

- ☐ Animals treated with vehicle
- ☐ Animals treated with MSC-conditioned media
- ☐ Animals treated with MSCs
- ☐ Animals treated with EVs derived from another cell type (e.g. fibroblasts)
- ☐ Other (add below)

Permanently add an answer to this question ()

61. Sample size of comparator/control group

...

62. Mean of outcome (comparator/control group)

...

63. Standard deviation of outcome (comparator/control group)

...

64. How many experimental groups were there (i.e. groups treated with MSC-EVs)?

- ☐ 1
- ☐ 2
- ☐ 3

Permanently add an answer to this question ()

Clear Response ()

65. What was the experimental group 1?

- ☐ Animals treated with unmodified MSC-EVs
- ☐ Animals treated with EVs derived from pre-treated MSCs (specify, e.g. hypoxic culture conditions)
- ☐ Animals treated with EVs derived from modified MSCs (specify, e.g. transfected MSCs)
- ☐ Animals treated with modified EVs (specify, e.g. transfected EVs)
- ☐ Animals treated with EVs co-administered with another treatment (specify, e.g. antibiotics)
- ☐ Other (add below)

Permanently add an answer to this question ()

66. Sample size (experimental group 1)

...

67. Mean of outcome (experimental group 1)

...

68. Standard deviation of outcome (experimental group 1)

...

69. Latest timepoint at which outcome was measured

...

☒ Inflammation (ALI)

70. How was this parameter measured?

☒ Number of inflammatory cells

71. What was the comparator/control group?

- ☐ Animals treated with vehicle
- ☐ Animals treated with MSC-conditioned media
- ☐ Animals treated with MSCs
- ☐ Animals treated with EVs derived from another cell type (e.g. fibroblasts)
- ☐ Other (add below)

Permanently add an answer to this question ()

72. Sample size of comparator/control group

...

73. Mean of outcome (comparator/control group)

...

74. Standard deviation of outcome (comparator/control group)

...

75. How many experimental groups were there (i.e. groups treated with MSC-EVs)?

- ☐ 1
- ☐ 2
- ☐ 3

Permanently add an answer to this question ()

Clear Response ()

76. What was the experimental group 1?

- ☐ Animals treated with unmodified MSC-EVs
- ☐ Animals treated with EVs derived from pre-treated MSCs (specify, e.g. hypoxic culture conditions)
- ☐ Animals treated with EVs derived from modified MSCs (specify, e.g. transfected MSCs)
- ☐ Animals treated with modified EVs (specify, e.g. transfected EVs)
- ☐ Animals treated with EVs co-administered with another treatment (specify, e.g. antibiotics)
- ☐ Other (add below)

Permanently add an answer to this question ()

77. Sample size (experimental group 1)

...

78. Mean of outcome (experimental group 1)

...

79. Standard deviation of outcome (experimental group 1)

...

80. Latest timepoint at which outcome was measured

...

☒ Cytokines levels in BALF

Units?

...

What was the comparator/control group?

- ☐ Animals treated with vehicle
- ☐ Animals treated with MSC-conditioned media
- ☐ Animals treated with MSCs
- ☐ Animals treated with EVs derived from another cell type (e.g. fibroblasts)
- ☐ Other (add below)

Permanently add an answer to this question {}

Sample size of comparator/control group

...

Mean of outcome (comparator/control group)

...

Standard deviation of outcome (comparator/control group)

...

86. How many experimental groups were there (i.e. groups treated with MSC-EVs)?

- ☐ 1
- ☐ 2
- ☐ 3

Permanently add an answer to this question {}

Clear Response {}

87. What was the experimental group 1?

- ☐ Animals treated with unmodified MSC-EVs
- ☐ Animals treated with EVs derived from pre-treated MSCs (specify, e.g. hypoxic culture conditions)
- ☐ Animals treated with EVs derived from modified MSCs (specify, e.g. transfected MSCs)
- ☐ Animals treated with modified EVs (specify, e.g. transfected EVs)
- ☐ Animals treated with EVs co-administered with another treatment (specify, e.g. antibiotics)
- ☐ Other (add below)

Permanently add an answer to this question {}

Sample size (experimental group 1)

...

Mean of outcome (experimental group 1)

...

Standard deviation of outcome (experimental group 1)

...

Latest timepoint at which outcome was measured

...

☐ Other (add below)

Permanently add an answer to this question {}

☒ Pulmonary hypertension (BPD)

92. How was this parameter measured?

☒ Right ventricle hypertrophy (Fulton's index)

93. What was the comparator/control group?

- ☐ Animals treated with vehicle
- ☐ Animals treated with MSC-conditioned media
- ☐ Animals treated with MSCs
- ☐ Animals treated with EVs derived from another cell type (e.g. fibroblasts)
- ☐ Other (add below)

Permanently add an answer to this question ()

94. Sample size of comparator/control group

...

95. Mean of outcome (comparator/control group)

...

96. Standard deviation of outcome (comparator/control group)

...

97. How many experimental groups were there (i.e. groups treated with MSC-EVs)?

- ☐ 1
- ☐ 2
- ☐ 3

Permanently add an answer to this question ()

Clear Response ()

98. What was the experimental group 1?

- ☐ Animals treated with unmodified MSC-EVs
- ☐ Animals treated with EVs derived from pre-treated MSCs (specify, e.g. hypoxic culture conditions)
- ☐ Animals treated with EVs derived from modified MSCs (specify, e.g. transfected MSCs)
- ☐ Animals treated with modified EVs (specify, e.g. transfected EVs)
- ☐ Animals treated with EVs co-administered with another treatment (specify, e.g. antibiotics)
- ☐ Other (add below)

Permanently add an answer to this question ()

99. Sample size (experimental group 1)

...

100. Mean of outcome (experimental group 1)

...

101. Standard deviation of outcome (experimental group 1)

...

102. Latest timepoint at which outcome was measured

...

☐ Other (add below)

Permanently add an answer to this question ()

☒ Pulmonary vascular remodeling (BPD, PAH)

103. How was this parameter measured?

☒ Medial thickness index (BPD, PAH)

104. What was the comparator/control group?

- ☐ Animals treated with vehicle
- ☐ Animals treated with MSC-conditioned media
- ☐ Animals treated with MSCs
- ☐ Animals treated with EVs derived from another cell type (e.g. fibroblasts)
- ☐ Other (add below)

Permanently add an answer to this question ()

105. Sample size of comparator/control group

...

106. Mean of outcome (comparator/control group)

...

107. Standard deviation of outcome (comparator/control group)

...

108. How many experimental groups were there (i.e. groups treated with MSC-EVs)?

- ☐ 1
- ☐ 2
- ☐ 3

Permanently add an answer to this question ()

Clear Response ()

109. What was the experimental group 1?

- ☐ Animals treated with unmodified MSC-EVs
- ☐ Animals treated with EVs derived from pre-treated MSCs (specify, e.g. hypoxic culture conditions)
- ☐ Animals treated with EVs derived from modified MSCs (specify, e.g. transfected MSCs)
- ☐ Animals treated with modified EVs (specify, e.g. transfected EVs)
- ☐ Animals treated with EVs co-administered with another treatment (specify, e.g. antibiotics)
- ☐ Other (add below)

Permanently add an answer to this question ()

110. Sample size (experimental group 1)

...

111. Mean of outcome (experimental group 1)

...

112. Standard deviation of outcome (experimental group 1)

...

113. Latest timepoint at which outcome was measured

...

☒ Pulmonary vessel density (BPD)

Units?

...

What was the comparator/control group?

- ☐ Animals treated with vehicle
- ☐ Animals treated with MSC-conditioned media
- ☐ Animals treated with MSCs
- ☐ Animals treated with EVs derived from another cell type (e.g. fibroblasts)
- ☐ Other (add below)

Permanently add an answer to this question ()

Sample size of comparator/control group

...

Mean of outcome (comparator/control group)

...

Standard deviation of outcome (comparator/control group)

...

119. How many experimental groups were there (i.e. groups treated with MSC-EVs)?

- ☐ 1
- ☐ 2
- ☐ 3

Permanently add an answer to this question ()

Clear Response ()

120. What was the experimental group 1?

- ☐ Animals treated with unmodified MSC-EVs
- ☐ Animals treated with EVs derived from pre-treated MSCs (specify, e.g. hypoxic culture conditions)
- ☐ Animals treated with EVs derived from modified MSCs (specify, e.g. transfected MSCs)
- ☐ Animals treated with modified EVs (specify, e.g. transfected EVs)
- ☐ Animals treated with EVs co-administered with another treatment (specify, e.g. antibiotics)
- ☐ Other (add below)

Permanently add an answer to this question ()

Sample size (experimental group 1)

...

Mean of outcome (experimental group 1)

...

Standard deviation of outcome (experimental group 1)

...

Latest timepoint at which outcome was measured

...

☐ Other (add below)

Permanently add an answer to this question ()

☐ No

Form Preview

☒ Survival

Clear Response ()

Units?

...

What was the comparator/control group?

- ☐ Animals treated with vehicle
- ☐ Animals treated with MSC-conditioned media
- ☐ Animals treated with MSCs
- ☐ Animals treated with EVs derived from another cell type (e.g. fibroblasts)
- ☐ Other (add below)

Permanently add an answer to this question ()

Sample size of comparator/control group

...

Mean of outcome (comparator/control group)

...

Standard deviation of outcome (comparator/control group)

...

130. How many experimental groups were there (i.e. groups treated with MSC-EVs)?

- ☐ 1
- ☐ 2
- ☐ 3

Permanently add an answer to this question ()

Clear Response ()

131. What was the experimental group 1?

- ☐ Animals treated with unmodified MSC-EVs
- ☐ Animals treated with EVs derived from pre-treated MSCs (specify, e.g. hypoxic culture conditions)
- ☐ Animals treated with EVs derived from modified MSCs (specify, e.g. transfected MSCs)
- ☐ Animals treated with modified EVs (specify, e.g. transfected EVs)
- ☐ Animals treated with EVs co-administered with another treatment (specify, e.g. antibiotics)
- ☐ Other (add below)

Permanently add an answer to this question ()

Sample size (experimental group 1)

...

Mean of outcome (experimental group 1)

...

Standard deviation of outcome (experimental group 1)

...

Latest timepoint at which outcome was measured

...

☐ Other (add below)

Permanently add an answer to this question ()

136. Adverse events?

☐ Yes (specify)☐ No

Appendix D – Supplemental Material for Chapter 6

Supplemental File D1 – Animal Wellness Sheet

Animal Clinical Observation Sheet

A – PROTOCOL NUMBER/PROCEDURE #OHRI-3352							
Investigator Duncan Stewart/ Manoj Lalu	Daytime:	Out-of-Hours:	E-mail:				
Laboratory Personnel Damian Chwastek	Daytime:	Out-of-Hours:	E-mail:				
B – CAGE ID				C – ANIMAL ID			
Experimental procedure: Intratracheal VSV-CoV2-S virus				Date/time start:			
				Date/time start:			
Experimental endpoint:				Date/time endpoint:			
Please note: The observations below are meant to capture information from all animals in the cage. In the event that an individual animal is not recovering as expected, please advise ACVS staff immediately. Please also identify the animal number and note more specific health information on the back of the page. Descriptions of the murine sepsis score (MSS) is attached.							
D – ANIMAL OBSERVATION							
Post- viral administration	1 hr Visual	3 hr Visual	6 hr Wellness	24 hr Wellness	48 hr Wellness	72 hr Wellness	96 hr Wellness
Date							
Time							
Body weight (g) (as required)							
Appearance (0- 4)							
Level of Consciousness (0- 4)							
Activity (0- 4)							
Response to stimulus (0- 4)							
Eyes (0- 4)							
Respiration rate (0- 4)							
Respiration quality (0- 4)							
Facial Grimace Score (0- 2)							
Total Score:							
**If total score is > 21 or respiration rate/quality = 4, recheck in 1 hour and contact research staff regarding end point advisement							
Temperature			☐	☐	☐	☐	☐

E - TREATMENT							
Ketamine/Xylazine i.p. given prior to procedure (120/6 mg/kg)							
Initials							
F - ACC ADDITIONS OR EXCLUSIONS FROM ENDPOINTS: Temperature alone is not to be a criterion for euthanasia							
G – ACC EUTHANASIA METHOD FOR ENDPOINTED ANIMALS: CO2 - Please contact Damian Chwastek prior to euthanizing animal							
H – DISPOSAL OF CARCASSES							
☐ Refrigerate (-4°C) ☐ Fixation (10% Buffered Formalin; whole body) ☐ Freeze (-20°C) ☒ Discard ☒ Other (describe) Please email Damian Chwastek							

D – ANIMAL OBSERVATION							
Post- viral administration	Day 5 Wellness	Day 6 Wellness	Day 7 Wellness	Day 8 Wellness	Day 9 Wellness	Day 10 Wellness	
Date							
Time							
Body weight (g) (as required)							
Appearance (0- 4)							
Level of Consciousness (0- 4)							
Activity (0- 4)							
Response to stimulus (0- 4)							
Eyes (0- 4)							
Respiration rate (0- 4)							
Respiration quality (0- 4)							
Facial Grimace Score (0- 2)							
Total Score:							
**If total score is > 21 or respiration rate/quality = 4, recheck in 1 hour and contact research staff regarding end point advisement							
Temperature	☐	☐	☐	☐	☐	☐	☐
E - TREATMENT							

Ketamine/Xylazine i.p. given prior to procedure (120/6 mg/kg)							
Initials							
F - ACC ADDITIONS OR EXCLUSIONS FROM ENDPOINTS: Temperature alone is not to be a criterion for euthanasia							
G – ACC EUTHANASIA METHOD FOR ENDPOINTED ANIMALS: CO2 - Please contact Damian Chwastek prior to euthanizing animal							
H – DISPOSAL OF CARCASSES ☐ Refrigerate (-4°C) ☐ Fixation (10% Buffered Formalin; whole body) ☐ Freeze (-20°C) ☒ Discard X Other (describe) Please email Damian Chwastek							

Variable	Score	Description
Appearance	0 1 2 3 4	- Coat is smooth - Patches of hair piloerected - Majority of back is piloerected - Piloerection may or may not be present, mouse appears "puffy" - Piloerection may or may not be present, mouse appears emaciated
Level of consciousness	0 1 2 3 4	- Mouse is active - Mouse is active but avoids standing upright - Mouse activity is noticeably slowed. The mouse is still ambulant. - Activity is impaired. Mouse only moves when provoked, movements have a tremor - Activity severely impaired. Mouse remains stationary when provoked, with possible tremor
Activity	0 1 2 3 4	- Normal amount of activity. Mouse is any of: eating, drinking, climbing, running, fighting - Slightly suppressed activity. Mouse is moving around bottom of cage - Suppressed activity. Mouse is stationary with occasional investigative movements - No activity. Mouse is stationary - No activity. Mouse experiencing tremors, particularly in the hind legs
Response to stimulus	0 1 2 3 4	- Mouse responds immediately to auditory stimulus or touch - Slow or no response to auditory stimulus; strong response to touch (moves to escape) - No response to auditory stimulus; moderate response to touch (moves a few steps) - No response to auditory stimulus; mild response to touch (no locomotion) - No response to auditory stimulus. Little or no response to touch. Cannot right itself if pushed over
Eyes	0 1 2 3 4	- Open - Eyes not fully open, possibly with secretions - Eyes at least half closed, possibly with secretions - Eyes half closed or more, possibly with secretions - Eyes closed or milky
Respiration rate	0 1 2 3 4	- Normal, rapid mouse respiration - Slightly decreased respiration (rate not quantifiable by eye) - Moderately reduced respiration (rate at the upper range of quantifying by eye) - Severely reduced respiration (rate easily countable by eye, 0.5 s between breaths) - Extremely reduced respiration (>1 s between breaths)
Respiration quality	0 1 2 3 4	- Normal - Brief periods of laboured breathing - Laboured, no gasping - Laboured with intermittent gasps - Gasping

Facial expressions indicating pain in rodents are shown:

1. Orbital tightening
2. Nose bulge/cheek flattening
3. Ear position changes
4. Whisker position changes

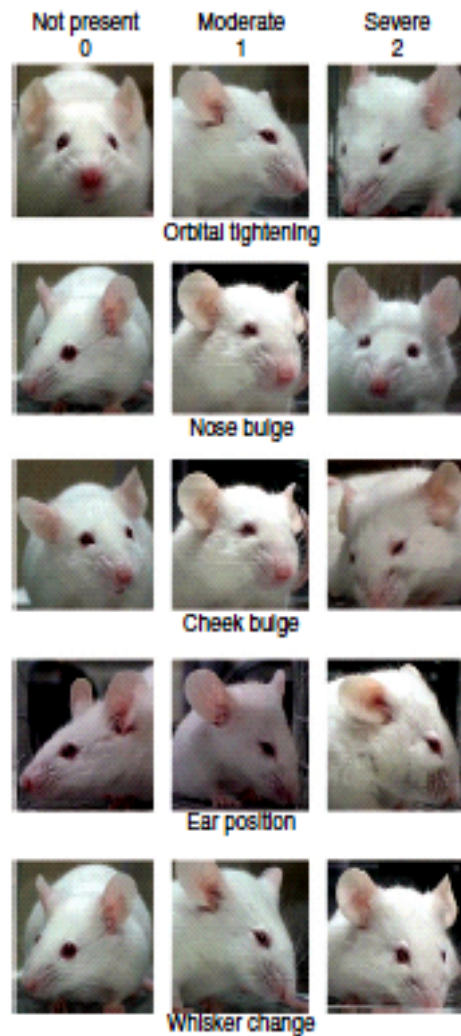


Figure 1 | In the MGS, intensity of each feature is coded on a three-point scale. For each of the five features, images of mice exhibiting behavior corresponding to the three values are shown.

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