

# “Good things come in small packages”: application of exosome-based therapeutics in neonatal lung injury

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Infants born at very low gestational age contribute disproportionately to neonatal morbidity and mortality. Advancements in antenatal steroid therapies and surfactant replacement have favored the survival of infants with ever-more immature lungs. Despite such advances in medical care, cardiopulmonary and neurological impairment prevail in constituting the major adverse outcomes for neonatal intensive care unit survivors. With no single effective therapy for either the prevention or treatment of such neonatal disorders, the need for new tools to treat and reduce risk of further complications associated with extreme preterm birth is urgent. Mesenchymal stem/stromal cell (MSC)-based approaches have shown promise in numerous experimental models of lung injury relevant to neonatology. Recent studies have highlighted that the therapeutic potential of MSCs is harnessed in their secretome, and that the therapeutic vector therein is represented by the exosomes released by MSCs. In this review, we summarize the development and significance of stem cell-based therapies for neonatal diseases, focusing on preclinical models of neonatal lung injury. We emphasize the development of MSC exosome-based therapeutics and comment on the challenges in bringing these promising interventions to clinic.

Infants born at very low gestational age contribute disproportionately to neonatal morbidity and mortality, and are at increased risk of developing several diseases including, periventricular leukomalacia, retinopathy of prematurity, hypoxic-ischemic encephalopathy, and bronchopulmonary dysplasia (BPD) (1–3). With no single effective therapy for either the prevention or treatment of such neonatal disorders, the need for new tools to treat and reduce risk of further complication associated with extreme preterm birth is urgent.

Recently, Stoll *et al.* (1) investigated the 20-year survival and outcome trend of >34,000 preterm infants, and found that the frequency of several morbidities associated with prematurity has decreased, yet between 2009 and 2012 the

prevalence of BPD had increased across infants of 22–27 weeks of gestational age. Consequently, BPD is the most common complication of preterm birth, affecting ~35% of infants born at ≤28 week's gestation. This trend is likely attributed to advancements in medical care that favor the survival of extremely preterm infants. As a result, the modern-day neonatal intensive care unit witnesses the presentation of ever-more immature lungs, complicating the challenge of preventing and treating neonatal lung injury (4).

BPD is a multifactorial chronic lung disorder that occurs almost exclusively in preterm infants receiving supplemental oxygen and mechanical ventilation. It is characterized by lung growth arrest, alveolar simplification, impaired blood vessel development, and abnormal pulmonary function (5). Infants with BPD exhibit abnormalities in lung function that often begin at birth and persist through infancy, childhood, and into adolescence. In turn, young adults who were former BPD infants may present with increased incidence of reactive airway disease and premature lung dysfunction with “emphysema-like” features of lung disease (6,7). Moreover, the development of pulmonary hypertension in moderate-to-severe cases of BPD is linked to increased mortality (8,9). Collectively, BPD is no longer considered a disease specific for the neonatal period, but a multifactorial condition with lifelong consequences.

## LACK OF THERAPIES FOR BRONCHOPULMONARY DYSPLASIA

Current neonatal intensive care unit treatment strategies aim to not only support the survival of the preterm infant but also minimize further lung injury and facilitate recovery (see for a recent review ref. (10)). Aside from the notable improvements in ventilation strategies, surfactant supplementation, and the use of corticosteroids, there has been a lack of new therapies that have had a marked effect in preventing/treating BPD. Currently, active research avenues are exploring the most efficient method of surfactant administration; synthetic vs. animal-derived surfactant; time and dose optimization for corticosteroid treatment; and further advancements in

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ventilation techniques (11). Despite the necessity for continuing optimization, it is fair to speculate that classical pharmacological therapies and “subtle” technological improvements may have only a modest impact on BPD outcomes. Moreover, in multifactorial disorders like BPD, it is unlikely that therapies targeting a single pathway will achieve a significant clinical impact. Indeed, inhaled nitric oxide (12) and vitamin A supplementation (13) are just some of the alternative drug approaches that have failed to consistently produce effective clinical outcomes. Thus, there is a burgeoning need for novel therapeutic approaches to restore homeostasis and allow regular developmental growth. Stem cell-based therapies represent one such approach, which holds great promise. Herein, we will comment on the development of mesenchymal stem/stromal cell (MSC)-based therapies in experimental models of neonatal lung injury, focusing on MSC-exosome therapeutics.

#### EARLY EVIDENCE OF DEPLETION AND/OR DYSREGULATION OF ENDOGENOUS PROGENITOR/STEM CELL POPULATIONS IN NEONATAL LUNG INJURY

It is widely acknowledged that the preterm infant is exposed to several risk factors that result in tissue injury across multiple organs, but an understanding of the mechanisms underlying tissue simplification is incomplete. Common mechanisms of pathogenesis include inflammation, infection, ischemia–reperfusion, and nitro-oxidative stress. In addition, genetic susceptibility and nutritional deprivation play a crucial role in exacerbating injury (14,15). The combination of such risk factors may lead to arrest of normal organ growth. Emerging evidence suggests that loss of endogenous stem/progenitor cells required for normal cell differentiation and tissue repair may also underlie the pathobiology of such injury (14,16,17).

The lungs are equipped with an arsenal of endogenous stem/progenitor cell populations that are readily mobilized upon cellular injury, proliferating and differentiating to actively repair and regenerate injured tissue (recently reviewed in refs (15,18,19)). Previous studies have found that exposure to hyperoxia (80% O<sub>2</sub>) reduces endothelial progenitor cell (defined as Cd45-/Sca-1<sup>+</sup>/Cd133<sup>+</sup>/Vegfr-2<sup>+</sup>) levels in the circulation, bone marrow, and lungs of neonatal mice (20).

In the clinical setting, Bozyk *et al.* showed that lung-resident MSCs isolated from the tracheal aspirates of preterm neonates with respiratory distress, demonstrating increased expression of proinflammatory cytokines (21). In accordance, Popova *et al.* found the presence of MSCs in the tracheal aspirates of preterm infants (<33 weeks gestational age), which was found to be significantly associated with subsequent development of BPD (22). Although the biological role of tracheal aspirate MSCs in lung homeostasis and repair remains unclear, these studies imply that both depletion and/or dysfunction of endogenous progenitor/stem cells can increase the risk of BPD.

Lung-resident MSCs are a multipotent stem cell population. It is believed that such tissue-resident stem cells are key regulators of pulmonary homeostasis and play an important role in regulating the lung immune and repair responses in processes such as inflammation, angiogenesis, and fibrosis (15,18,19). Previous studies have shown that treatment of adult mice with bleomycin was associated with a significant loss of endogenous lung MSCs, fibrosis, inflammation, and pulmonary arterial hypertension. Interestingly, transplantation of isolated “control” lung MSCs attenuated the bleomycin-associated pathology and mitigated the development of pulmonary arterial hypertension via restoration of the effector T-cell response (23).

However, there are “two sides to every coin”. In addition to their reparative properties, studies have shown that lung MSCs under specific conditions can mediate pathogenic changes in the lung, a process governed by their immediate microenvironment. For example, Chow *et al.* (24) reported that increased oxidative stress, induced via a targeted depletion of extracellular superoxide dismutase, modulated the programming of resident lung MSCs, causing them to contribute to pulmonary microvascular remodeling. Furthermore, it was recently shown that transforming growth factor- $\beta$  expression within the lungs of premature infants stimulates MSCs to differentiate into myofibroblasts. Such reports highlight the importance of the microenvironment in regulating MSC function (25).

The bulk of the literature surrounding pulmonary progenitor/stem cells is derived from preclinical research that often involves mice. Compared with the mouse, less information is known about endogenous progenitor/stem cell populations in human lung. However, findings from preclinical studies and small clinical cohorts to date provide early evidence that loss and/or dysregulation of endogenous progenitor/stem cell populations that facilitate growth, development, and repair of developing organs could underpin the pathophysiology of neonatal disorders such as BPD. Collectively, such findings provided a foundation to explore stem cell-based therapies to treat and/or prevent neonatal lung injury. Indeed, further studies and continuing progress in this field is essential for our understanding of the molecular mechanisms susceptible to pulmonary perinatal/neonatal stress.

#### MSC THERAPY IN BRONCHOPULMONARY DYSPLASIA

Transplantation of different stem cell types, including endothelial colony-forming cells and human amnion epithelial cells have shown promise in preclinical models for the prevention and/or treatment of BPD and other major sequelae of prematurity. Such studies have also demonstrated profound anti-inflammatory, neuroprotective, and functional benefits in preclinical models of neonatal hypoxia–ischemia, cerebral palsy, and stroke (see for recent reviews refs (15,26–32)). Herein, we will focus on therapeutic applications using MSCs and their secreted products.

MSCs are nonhematopoietic adult stem cells that can be successfully propagated *in vitro*. Methods for isolating MSCs

from several human tissues, including bone marrow (BMSCs), Wharton's jelly, umbilical cord blood, and adipose tissue are well documented (33,34). With no known specific MSC marker, MSCs are defined by their adherence to plastic, the demonstration of *in vitro* differentiation potential to mesodermal lineage, and conformity to a well-characterized surface marker panel (35).

MSC treatment was shown to be efficacious in a number of animal models of lung disease, (reviewed in ref. (30)). Using experimental models of BPD, we (36) and others (37) have shown that MSC treatment blunts hyperoxia-induced lung inflammation, improves lung architecture, ameliorates vascular remodeling, improves exercise capacity, and increases survival rates. Promising preclinical data (reviewed in ref. (31)) and the demonstration of safety in several clinical trials on MSC treatment in adult lung diseases (38,39) have recently brought MSC therapy into clinical development for newborn lung disease. Specifically, in a single-center, phase I dose-escalation feasibility trial, nine preterm infants ( $25.3 \pm 0.9$  weeks of gestational age) who were predicted to be at highest risk of BPD were enrolled and treated with a bolus endotracheal dose of allogeneic human umbilical cord blood MSCs ( $10\text{--}20$  million cells/kg) at  $10.4 \pm 2.6$  postnatal days. MSC therapy was well tolerated, the trial demonstrated feasibility and short-term safety in preterm neonates and phase II trials are now in progress. Although functional end points are beyond the scope of phase I clinical trials, it was noticed that MSC administration was associated with a reduction in inflammatory markers from baseline in the tracheal aspirates of treated infants and lower BPD severity from the historical case-matched comparison group (40).

It is important to note that, although clinical trials are progressing, we lack complete understanding of the mechanism(s) underlying the therapeutic properties of MSCs. Earlier hope that MSCs could repair lung tissue by engrafting with high efficiency in the injured lung and transdifferentiating to pneumocytes was subsequently proven to be rather premature and claims supporting it were based on equivocal evidence.

The substantial physiologic improvements in the recipient lung following MSC transplantation, in the absence of significant engraftment of donor cells, has led to the generally accepted notion that the MSC therapeutic action is mainly through paracrine mechanisms (36,41,42). In an experimental model of "severe" hyperoxia-induced BPD, we previously assessed the therapeutic capacity of BMSCs and their respective secretome in parallel. Interestingly, we found that treatment with the BMSC secretome (specifically BMSC-conditioned media) provided a superior therapeutic capacity over MSCs themselves, providing greater protection in preventing alveolar simplification and preserving the lung architecture (36).

In a different study using a similar "severe" model of hyperoxia-induced BPD, our group demonstrated that a bolus dose of MSC-conditioned media, delivered intravenously after 14 days of hyperoxia exposure (75% O<sub>2</sub>), blunted lung inflammation, reversed pulmonary hypertension and vascular

pruning, ameliorated lung fibrosis, and improved lung architecture (43). In accordance, the Thébaud laboratory also demonstrated that the therapeutic capacity of MSCs was harnessed in their secretome (44). They noted that the BMSC-conditioned media accelerated wound healing and enhanced endothelial cord formation *in vitro* and that human umbilical cord MSC-conditioned media afforded short- and long-term therapeutic benefits without adverse lung effects in a rat model of hyperoxia-induced BPD (37,45,46).

Although the paracrine mode of MSC action is a generally accepted notion, we have not yet clearly defined the precise bioactive moiety responsible for their therapeutic efficacy. The complex MSC secretome contains proteins of diverse functions, nucleic acids, morphogens, free fatty acids, genetic information (including small noncoding RNA), and membrane components (47). Over the last several years a number of these bioactive molecules have been reported to represent the active component(s) of the MSC secretome, but, in general, studies attempting to recapitulate the effects of MSC-conditioned media *in vivo* through the administration of the isolated candidate molecule did not yield consistent and reproducible results. This led to the hypothesis that the therapeutic vector of the MSC secretome was represented by moieties of higher molecular complexity, and in particular, the subclass of extracellular vesicles (EVs) termed exosomes. Recent validation of this hypothesis in several preclinical studies creates the promise that exosomes may represent a novel and exciting approach to treating neonatal lung diseases.

#### EXTRACELLULAR VESICLES: NOMENCLATURE AND CLASSIFICATION

Exosomes are submicron (~30–150 nm), plasma membrane-enclosed EVs. Originally assumed to represent a mechanism for the cell to jettison unwanted moieties, the secretion of EVs, and particularly the subclass generated via the endosomal sorting machinery, is now considered to represent an effective method for cell-to-cell communication via the transfer of cellular components (15,48–50). As our understanding of exosome biogenesis has improved, it has become apparent that during their formation, exosomes can associate with an assortment of "bioactive" cargo from their parental cell. In turn, exosomes are at the forefront of active research themes for diagnostic, prognostic, and therapeutic applications across multiple disciplines (51).

As the consequence of a relatively new multidisciplinary research field, the nomenclature and classification of these vesicles remains abstract. Typically, EVs are categorized into three subclasses: exosomes (~30–150 nm in diameter), microvesicles (~100 nm–1 μm in diameter), and apoptotic bodies (>1 μm). These subclasses of vesicles were grouped based on their biogenesis and biophysical properties (such as size, density, and predominant protein markers). Exosome biogenesis and nomenclature has been reviewed extensively (47,48,52–55).

With limitations in current technology, the field currently lacks the ability to analyze exosomes at a single vesicle level. Thus, it is becoming increasingly difficult to uphold one terminology over another. Arguably, as a consequence of no “gold-standard” methods for EV isolation, characterization, and quantification, and a concomitant concern within the field about the inappropriate application and interpretation of studies, the International Society for Extracellular Vesicles (ISEV) previously urged the widespread adoption of the generic term EV when referring to secreted vesicles (52). Using the “generic” EV terminology demands a complementary EV biophysical characterization (e.g., assessment of predominant protein markers, density, vesicle quantification, morphology, particle:protein ratio) to help specify the EV population in question. However, we argue that a comprehensive characterization is a quest that is seldom executed in previous/concurrent literature. Moreover, lack of EV characterization coupled with “poor” isolation methods that are prone to coisolate non-EV material often obfuscates the true potential and biological activity of EVs.

For the purposes of this review, we will just note that it can be particularly misleading to attempt to classify EV subpopulations by size alone, as isolation manipulations often break up larger EVs, generating an artificial heterogeneity in the smaller size subclasses. Herein, we will adopt the nomenclature chosen by the cited articles, and support nomenclature with their respective isolation methods where appropriate.

### MSC-EXOSOME THERAPY IN EXPERIMENTAL BRONCHOPULMONARY DYSPLASIA

The therapeutic capacity of MSC “exosomes” derived from different organs have been tested in various disease models, demonstrating a similar or even superior functional capacity to MSCs themselves, reducing myocardial infarction size (56,57), aiding repair of kidney injury (58), and orchestrating neurological protection by the transfer of microRNA (59). A summary of MSC-exosome-based approaches for the treatment of different disease models is provided in **Table 1**. Previously, our group was the first to demonstrate that exosomes mediate the cytoprotective effect of MSCs in the lung, using a mouse model of hypoxia-induced pulmonary hypertension (60). Here, administration of MSC exosomes, isolated by size exclusion chromatography and characterized through the presence of established exosome markers (including Alix and TSG101, Flotilin-1, and tetraspanins (CD81, CD9)) and transmission electron microscopy, protected against the elevation of right ventricular systolic pressure and the development of right ventricular hypertrophy after 3 weeks of hypoxic exposure (8% O<sub>2</sub>). Importantly, exosome-depleted conditioned media had no effect. BMSC-exosome treatment was also able to abrogate early hypoxic macrophage influx and downregulate hypoxia-activated inflammatory pathways, thus recapitulating the anti-inflammatory actions of MSCs (60,61).

More recently, our group assessed the effect of highly purified human MSC exosomes, isolated by density on iodixanol (OptiPrep) gradients, in an established neonatal mouse model of hyperoxia-induced BPD. Here, we subjected newborn FVB mice to hyperoxia (75% O<sub>2</sub>) for 7 days (postnatal days 1–7) and administered a bolus intravenous dose of MSC exosomes at postnatal day 4. We found that a single dose of purified human MSC exosomes (the amount produced by  $0.5 \times 10^6$  MSCs over 36 h under standard tissue culture conditions) significantly improved lung morphology and pulmonary development, decreased lung fibrosis, ameliorated pulmonary vascular remodeling, and restored the hyperoxia-induced loss of pulmonary blood vessels. Importantly, we found that MSC-exosome treatment improved lung function and alleviated associated pulmonary hypertension as assessed at postnatal day 42, long after the hyperoxic lung injury. The therapeutic efficacy of exosomes was comparable for preparations obtained from MSC cultures derived from either human bone marrow or human umbilical cord Wharton’s Jelly. This observation opens the possibility that Wharton’s jelly MSCs, a resource far more abundant and easily obtainable than BMSCs may expedite large-scale, good manufacturing practice production of MSC exosomes for upcoming clinical trials (62).

Mechanisms by which EVs and their respective vesicle subtypes induce their therapeutic effects remain incompletely understood. With beneficial effects in diverse disease models, it is reasonable to suggest that a single “bioactive” molecule/pathway cannot account for their entire therapeutic capacity. It is well reported that MSC-EVs harbor a wealth of bioactive cargo. We hypothesize that such “bioactive” cargo may synergistically play a role; however, with such heterogeneity in EV secretion, the precise bioactive ingredient maybe disease/model-specific.

Considering this, recent focus has been on the immunomodulatory capacity of MSC exosomes as an attempt to decipher the cellular mechanisms responsible for their therapeutic action. We recently showed that treatment with MSC exosomes (isolated by density gradient), blunts hyperoxia-induced inflammation, an effect that, at least in part, is mediated via modulation of lung macrophage phenotype. Specifically, Wharton’s jelly MSC exosomes suppressed the levels of proinflammatory (M1)-associated markers such as interleukin-6 and tumor necrosis factor- $\alpha$  and altered the expression proremodeling (M2) markers (such as Cd206 and Arginase-1) both *in vitro* and *in vivo* (62). In accordance, recent studies have shown that BMSC-EVs (isolated by differential ultracentrifugation, last step; 100,000 g for 90 min) modulates the macrophage transcriptome to favor an anti-inflammatory, M2-like phenotype, both *in vitro* and *in vivo* in a murine model of cardiotoxin-induced skeletal muscle injury (63). Although the above studies highlight a potent immunomodulatory effect following MSC-exosome treatment, one can only conclude an association. The precise “bioactive” modalities within the exosomes/EVs that are responsible for such effects remain unclear. A

**Table 1.** Summary of MSC “exosome”-based therapeutics

Disease	MSC-product “nomenclature”	Final isolation step	Dose assessment	Dose	Ref.
Bronchopulmonary dysplasia	Exosomes	Density cushion	Cell equivalent	0.5 × 10 <sup>6</sup>	(62)
Pulmonary hypertension	Exosomes	SEC	Protein	0.1–10 µg	(60)
Pulmonary hypertension	Exosomes	UC (100 K × g)	Protein	25 µg	(85)
Acute lung injury	Microvesicles	UC (100 K × g)	Cell equivalent	1.5 × 10 <sup>6</sup>	(64)
Silicosis	Exosomes	Sucrose gradient	Protein	40 µg	(65)
Pneumonia	Microvesicles	UC (100 K × g)	Cell equivalent	9 × 10 <sup>6</sup>	(86)
Myocardial infarction	Exosomes	ExoQuick	Cell equivalent	4 × 10 <sup>6</sup>	(87)
Myocardial infarction	EVs	UC (100 K × g)	Protein	80 µg	(88)
Myocardial infarction	Exosomes	ExoQuick	Protein	80 µg	(89)
Ischemia–reperfusion	Exosomes	HPLC	Protein	0.4 µg	(56)
Ischemia–reperfusion	Exosomes	HPLC	Protein	0.4–0.8 µg	(57)
Traumatic brain injury	EVs	Anion exchange chromatography	Protein	30 µg	(90)
Laser-induced retinal injury	Exosomes	UC (110 K × g)	Protein	10 µg	(91)
Optical nerve crush	Exosomes	UC (100 K × g)	ExoELISA	3 × 10 <sup>9</sup>	(92)
Stroke	EVs	UC (110 K × g)	Cell equivalent	2 × 10 <sup>6</sup>	(93)
Stroke	Exosomes	UC (100 K × g)	Protein	100 µg	(94)
Cardiotoxin injury	EVs	UC (100 K × g)	Protein	5 µg	(63)
Drug-induced liver injury	Exosomes	UC (100 K × g)	Protein	0.4 µg	(95)
Liver fibrosis	Exosomes	UC (100 K × g)	Protein	250 µg	(96)
Colitis	EVs	UC (100 K × g)	Protein	50–200 µg	(95)
Wound healing	Exosomes	UC (100 K × g)	Protein	160 µg	(99)
Wound healing	Exosomes	UC (120 K × g)	Protein	100 µg	(98)
Ischemia–reperfusion	Microvesicles	UC (100 K × g)	Protein	100 µg	(100)
Acute kidney injury	Microvesicles	UC (100 K × g)	Protein	100 µg	(101)

Abbreviations: ELISA, enzyme-linked immunosorbent assay; EV, extracellular vesicle; HPLC, high-performance liquid chromatography; MSC, mesenchymal stem/stromal cell; SEC, size exclusion chromatography; U, ultracentrifugation. UC: 100,000–120,000 g (100 K–120 K × g). ExoQuick and ExoELISA refer to a commercially available exosome isolation kit and CD63 capture (exosome) ELISA, respectively (Systems Biosciences, Palo Alto, CA). Table adapted from ref. (76).

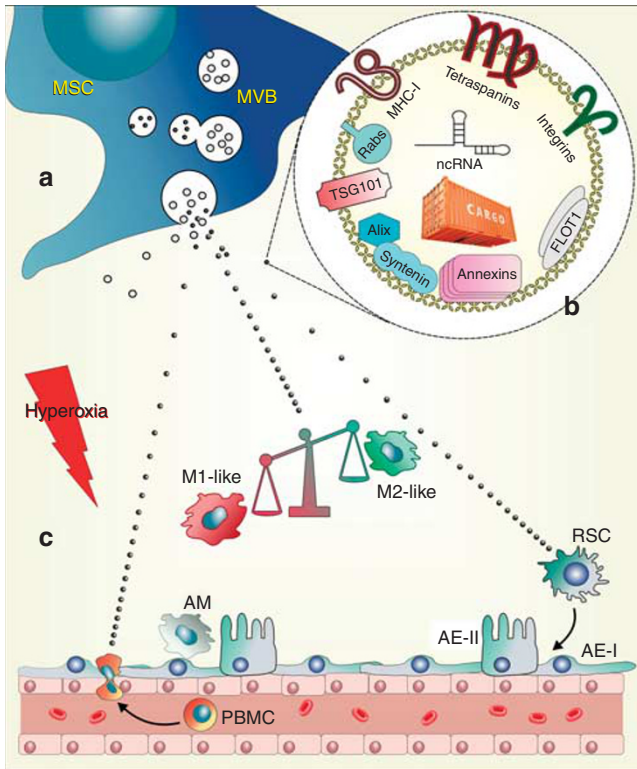
schematic representation of the immunomodulatory capacity of MSC exosomes is highlighted in **Figure 1**.

The concept that the therapeutic vector of MSCs is represented by exosomes is well supported by evidence, but we should be very cautious to assume that one specific mechanism could be operative in all disease models. With this in mind, human BMSC-EVs, isolated from conditioned media by sequential differential ultracentrifugation (final spin; 100,000 g for 2 h), have also been shown to exert anti-inflammatory and immunomodulatory effects on injured lung tissue, enhancing the resolution of pulmonary edema by upregulating sodium-dependent alveolar fluid clearance in experimental models of acute respiratory distress syndrome (64). Such reports suggest that the mechanism of action of MSCs may require cell-to-cell contact and mitochondrial transfer through tunneling nanotubes (41), or mitochondrial transfer through larger EV subclasses (> 500 nm in diameter), such as microvesicles (65). It is fair to assume that, depending on the particular injury and the specific microenvironment, MSCs may use a variety of mechanisms to repair injured tissue and restore pulmonary homeostasis, and

that the exosome merely represents one weapon in the arsenal of the MSC artillery.

### CHALLENGES IN BRINGING OF EXOSOME BASED-THERAPEUTICS

With such promising preclinical findings in numerous disease models, investigators are now tasked with developing a safe, feasible, and reproducible MSC-exosome therapy. However, the aim of bringing exosome-based therapies to clinical trials and assessing the actual potential of exosome-based therapeutics is met with significant challenges. Arguably, the major hurdles are the “poor” methodologies for exosome isolation and characterization and the absence of standardization in exosome purification. Akin to MSC therapy, difficulties in designing robust measures of exosome potency, and the challenges of industrial scale-up will complicate smooth transition to clinical development and must be resolved at an early phase. Herein, we comment on the potential for therapeutic application of MSC exosomes, outlining relevant issues to



**Figure 1.** Schematic of postulated therapeutic action of mesenchymal stem/stromal cell (MSC) exosomes in hyperoxia-induced bronchopulmonary dysplasia (BPD). (a) MSCs routinely generate exosomes in multivesicular bodies (MVBs) through the endocytic pathway. The majority of produced exosomes represents jettisoning of unwanted moieties by the cell and probably have no discernable function (larger gray symbols). Upon specific organismal or environmental cues, MSCs also produce a sub-population of exosomes that harbor the therapeutic activity (small black symbols). (b) The therapeutic exosomes harbor cell surface components arguably involved in targeting recipient cells (tetraspanins, integrins) or immunomodulation, such as major histocompatibility complex-I (MHC-I). They also contain molecules associated with the pathways of their biogenesis, such as Rabs, TSG101, Alix Syntenin, Annexins, and FLOT1. Their cargo includes small noncoding RNAs, but also macromolecular modules yet to be characterized. (c) The hyperoxic insult creates an inflammatory environment in the lung, which activates the alveolar macrophages (AMs) and recruits circulating monocytes (peripheral blood mononuclear cell (PBMC)) to the alveolar space. The main function of MSC exosomes is to induce a shift in macrophage polarization, tilting the balance from a destructive, M1-like inflammatory state to an anti-inflammatory, M2-like state. Additional actions of exogenously administered MSC exosomes could be the direct or indirect inhibition of PBMC recruitment to the injured lung and the direct or indirect enhancement of the activity of lung-resident stem cells (RSCs), leading to faster healing of injured tissue. AE-I and AE-II, alveolar epithelial cells type I and type II, respectively.

facilitate their development and provide guidance on the current challenges in bringing exosome-based therapies to clinic.

### Establishing a Preclinical Model

The choice of animal model is critical in the development of exosome-based therapeutics. Demonstrating efficacy at the preclinical stage is pivotal in determining the transition into

clinical development. For neonatal disorders such as BPD, several animal models have been developed and continue to be refined with the aim of mimicking the pathological pulmonary characteristics noted in lungs of neonates with BPD (66). Arguably BPD is most commonly modeled in mice. Mice have relatively short gestation times (~20 days), allowing studies that monitor saccular and alveolar lung development to be conducted relatively quickly. Importantly, mice delivered at term are in the saccular stage of lung development. Thus, the developmental stage of the mouse lung at birth resembles that in the human preterm neonate (born at 24 to 28 weeks gestation) (67), in turn making the newborn mouse an excellent model to study human developmental lung injury. However, it is important to note that, when term mice are born (in the saccular stage), these newborn rodent pups are competent for proper gas exchange and do not require oxygen supplementation as a life-saving intervention; this is in marked contrast to preterm human neonates (66,67).

The economic sustainability of the model is also important. In comparison with large animal models, small animals (e.g., rodents) require less exosomes per body weight. Thus, in addition to rodent models, future studies should consider larger animal models, such as the fetal lamb BPD model (68). Although the premature lamb will be larger than the gestational aged-matched preterm infant counterpart, alternative routes of administration (i.e., endotracheal) are possible, as is the ability to ventilate and investigate combination therapies, e.g., MSC exosomes combined with surfactant supplementation, thus mimicking more closely the current standard operating procedures in the neonatal intensive care unit.

### Characterization of Tissue/Cell Origin

Several open questions remain unanswered. Is the production of exosomes the same for all MSCs, independent of their tissue of origin? How do donor demographics and different cell culture conditions effect resultant MSC exosome? Such questions need to be addressed to facilitate the development of MSC-exosome therapeutics and standardize product formulations.

Donor-to-donor variability remains a prominent challenge. Indeed, MSCs obtained from older donors have slower proliferation and reduced differentiation potential *in vitro* (33,69). Borghesi *et al.* (70) screened different umbilical cord-MSC clones for acquired genomic imbalances during *in vitro* expansion. Using array-comparative genomic hybridization, they found that 2 out of 11 umbilical cord-MSC cultures carried genomic imbalances, generating genetic mosaicism at intermediate passages. Collectively, such uncertainties will likely affect the therapeutic capacity of MSCs, impacting on the characteristics and thus the potency of their resultant exosomes. It is therefore necessary to address these variables and exercise caution when designing MSC-based protocols.

## Exosome Heterogeneity

Although the field currently lacks exact tools to distinguish vesicles from different routes of biogenesis, recent studies have shown that MSCs release different exosome sub-populations that differ in biophysical, proteomic, and RNA repertoires. Specifically, Kowal *et al.* (71) found that large-, medium-, and small-sized EVs can be isolated by sequential centrifugation steps. Among the small EVs (where exosomes should fractionate), four subcategories were defined by their degree of CD63, CD9, and CD81 tetraspanin enrichment. It is fair to speculate that such exosome subtypes will mediate different effects on target cells. Thus, separation techniques that can distinguish between different exosome and/or small EV subtypes may help identify the functional sub-population and enrich for the active species. This will not only result in more potent preparations, but the resultant homogenous population will facilitate the deciphering of the molecular mechanisms underlying exosome function on their targets.

## Assessing Exosome Potency Assay/Dose Evaluation

Determining the optimal tool to reliably assess exosome dose, the appropriate time window for exosome administration, dosing frequency, and the most effective route of administration to achieve maximal therapeutic benefits without adverse effects are significant issues to resolve. Such issues will be disease/model-specific. However, with no standardized method to quantify exosomes, investigators currently rely on several different methods to assess exosome dosage. Typical quantitative methods include reporting cell equivalents, protein concentration, and/or specialized quantitative analytical measurements by instruments such as dynamic light scattering, tunable resistive pulse sensing and nanoparticle tracking analysis, with each technique subject to their own advantages and limitations. Beyond the scope of this review, exosome quantification methods are discussed in the following reviews (72–75).

An exosome potency assay would be a valuable tool in overcoming the inconsistencies in batch-to-batch variation and could also bypass the limitations surrounding current exosome quantification methods. Investigators could consider using an exosome potency assay to standardize practices and minimize biological variation between MSC-exosome preparations. To date, candidate MSC-exosome potency assays are arguably centered around T-cell proliferation and/or macrophage polarization assays (76). Importantly, the chosen exosome potency assay needs to be fit for purpose, employ relevant functional end points, and demonstrate a clear correspondence between *in vitro* potency (as measured by the chosen assay) and *in vivo* efficacy. The examples of assays above are based on the immunomodulatory properties of MSC exosomes and they can return a metric of this property. This metric could be proportional to *in vivo* efficacy, as it appears that one of the major effects of exosome treatment in the lung models of disease is indeed immunomodulation. Nevertheless, this represents an extreme oversimplification of the issue, and the establishment of a proper exosome potency

assay based on function will require careful design and even more careful validation. In the interim, in-depth exosome characterization may establish correlations between efficacy and specific exosome markers (e.g., expression of exosomal protein marker TSG101 or CD63). In turn, subsequent surrogate markers of exosome potency/dose maybe initially considered. Such “fingerprinting assays” may provide quality control and batch-to-batch consistency (77).

## Lung-on-a-Chip and Organoid Platforms

Improving preclinical predictions of experimental drug responses is critical to minimize failures and optimize success in clinical trials. Recent advancements in cell biology, bioengineering, and microfluidics have enabled the development of functional models of human organs, coined “organ-on-chips” (78,79). In addition to gaining further insight into MSC-exosome mechanism-of-action, the application of technologies such as lung-on-a-chip could provide a powerful tool for assessing MSC-exosome potency and to support preclinical assays with greater predictive power. The lung-on-a-chip microdevice could incorporate pulmonary epithelium, endothelium, and immune cells in a microenvironment consisting of a culture medium or blood–air interface and mechanical stretching, mimicking conditions prevalent at the early stages of disease.

Under certain culture conditions, macrophages, basal, stromal, secretory, and type II cells can give rise to three-dimensional, self-organizing structures known as organoids. Although preclinical data shows that MSC-exosome therapy results in robust physiological benefits, the biodistribution and *in vivo* metabolic fate of MSC exosomes remains difficult to assess. In turn, just as target cell interactions remains unclear, the effect of experimental drugs, such as MSC-exosome therapy in “whole lung” systems would allow insight to “off-target” cellular effects (80). Although such technologies are aimed to closely mimic *in vivo* conditions, when using such platforms to assess the potency of experimental drugs, fit-for-purpose functional end points need to be established.

## In Vivo Metabolic Fate and Biodistribution

For future exosome therapeutic applications, technological advances are needed to help improve our understanding of the *in vivo* fate of exosomes. Numerous studies have assessed the biodistribution of exosomes in mice. Recently, Wiklander *et al.* (81) and others (82,83) labeled exosomes with near-infrared lipophilic dyes and tracked the exosomes *in vivo* fate following intravenous, intraperitoneal, or subcutaneous injection. They found that the route of administration and the dose dictated the *in vivo* biodistribution pattern. However, in addition to dye dilution via target cell fusion, a potential limitation of using lipophilic dyes to track exosomes is the disparity in half-life between exosomes and the dye. At present, with each labeling method harboring its advantages and limitations, a multimodal approach is encouraged.

### Manufacturing, Toxicology, and Safety

In comparison with cell-based therapies in regenerative medicine, MSC-exosome treatment is more attractive for clinical development. Reasons being are manifold. MSC exosomes are “perceived” to be less immunogenic than their parental cells, as assessed by lower amounts of major histocompatibility complex-II. This may make MSC exosomes less likely to invoke an immunological response in a foreign host. In addition, by replacing the administration of live cells with their secreted exosomes, many of the safety concerns and limitations associated with adoptive transplantation of viable replicating cells are also mitigated. Furthermore, emerging evidence suggests that exosomes are nebulizable and can be cryopreserved at  $-20^{\circ}\text{C}$  for 6 months with no loss to their biochemical activity.

The challenge of manufacturing a safe and reproducible medicinal product is complex. The regulatory landscape for exosome-based therapeutics is still evolving and typical toxicology testing approaches may not be considered appropriate for such biologic medicines because of complex bioactive properties that may include tumorigenicity and unknown off-target activities. With this in mind, we suggest that patients who receive MSC-exosome therapy are closely monitored for several years for the theoretical risk of tumor formation. Notwithstanding the theoretical potential of tumor formation, a recent meta-analysis looking at  $>1,000$  adult subjects has shown that there is no incidence of increased tumor risk up to 60 months after MSC (cell-therapy) treatment (84). To avoid unwanted immunological events and monitor off-target effect, upon advancing to clinical studies, investigators could monitor the immunogenicity of exosome-based therapies. Open questions include: Do MSC exosomes from different cell sources and donors possess the same immunomodulatory capacity? Are MSC exosomes susceptible to cytotoxicity by the endogenous T cells or natural killer cells of the host?

### SUMMARY

Stem cell approaches such as MSC therapy have shown promise in numerous preclinical models relevant to neonatology. Such studies provided the platform for early phase clinical trials to study the feasibility and safety of MSCs in preterm infants at risk of developing BPD. Recent data have demonstrated that the therapeutic capacity of MSCs is comprised in their secretome, and that the major therapeutic vector therein is represented by the exosomes the MSCs release. Indeed, exosome-based therapeutics may represent the next-generation drug delivery system, providing an unparalleled efficacy for the treatment of numerous diseases lacking efficient pharmacotherapy. However, their clinical application and development remains hampered by technological, mechanistic, and safety issues. Careful consideration of the key issues raised in this review may help in bringing exosome-based therapeutics a step closer to the clinic.

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