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Harnessing the Therapeutic Potential of the Stem Cell Secretome in Neonatal Diseases

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Abstract

Preterm birth and intra-partum related complications account for a substantial amount of mortality and morbidity in the neonatal period despite significant advancements in neonatal-perinatal care. Currently, there is a noticeable lack of curative or preventative therapies available for any of the most common complications of prematurity including bronchopulmonary dysplasia, necrotizing enterocolitis, intraventricular hemorrhage, periventricular leukomalacia and retinopathy of prematurity or hypoxic-ischemic encephalopathy, the main cause of perinatal brain injury in term infants. Mesenchymal stem/stromal cell-derived therapy has been an active area of investigation for the past decade and has demonstrated encouraging results in multiple experimental models of neonatal disease. It is now widely acknowledged that mesenchymal stem/stromal cells exert their therapeutic effects via their secretome, with the principal vector identified as extracellular vesicles. This review will focus on summarizing the current literature and investigations on mesenchymal stem/stromal cell-derived extracellular vesicles as a treatment for neonatal diseases and examine the considerations to their application in the clinical setting.

Introduction

Neonatal diseases including preterm birth complications and intrapartum-related events are the main causes of pediatric mortality < 5 years per World Health Organization 2000-2019[1]. Survival of premature infants has improved significantly over the past half century, most notably the extremely low gestational age neonates (ELGAN) who are at the highest risk for developing serious complications of prematurity that impact the central

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nervous system, cardiorespiratory system, and gastrointestinal tract[2,3]. The morbidities associated with prematurity unfortunately remain high despite advancement in neonatal-perinatal medicine and the use of antenatal steroids and pulmonary surfactant therapy[2,3]. In addition, the burden of the complications of prematurity are long-lasting and span into childhood and even adulthood[4-7]. At present, there are no curative therapies available for the most common complications of prematurity such as bronchopulmonary dysplasia (BPD), necrotizing enterocolitis (NEC), intraventricular hemorrhage (IVH), periventricular leukomalacia (PVL) and retinopathy of prematurity (ROP). The mainstay of treatment for these prematurity complications includes mechanical ventilation, supportive medical therapy and in extreme scenarios, surgical therapy. In contrast, the most common cause of morbidity in term infants is due to perinatal brain injury such as hypoxic ischemic encephalopathy (HIE), which does have a standard of care treatment but is non-curative and has a limited time frame for application. Most of these neonatal pathologies are challenging to develop treatments for given that the diseases occur in immature and developing organs and have complex underlying pathophysiological injury mechanisms (Figure 1). Therefore, there is an urgent need to develop new, safe, and effective therapies for the treatment and prevention of neonatal diseases. Stem cell, specifically mesenchymal stem/stromal cell (MSC) based therapies have emerged as a promising therapeutic option due to their inherent regenerative and immunomodulatory properties. Numerous preclinical studies of MSCs in several neonatal diseases have demonstrated favorable results and it is now known that the therapeutic effect of MSCs is mediated by a paracrine mechanism via the secretome with various studies identifying extracellular vesicles (EVs) as the therapeutic vector. EVs are lipid-bilayer enclosed microparticles, endogenously produced by cells that mediate intercellular communication via transfer of their diverse bioactive cargo. In this review, we will summarize recent advances in stem cell secretome therapy for intractable neonatal diseases in preclinical models, the ways in which we can harness their therapeutic potential and discuss the considerations and challenges to clinical applications in this vulnerable patient population.

The Evolution of Stem Cell Secretome Therapy

Stem cell therapy has been a promising field of investigation in medical science since the initial discovery in 1963 by Till and McCulloch of stem cells as undifferentiated cells with self-renewal and differentiation properties[8]. Since then, stem cells have found their way into clinical application with bone marrow (BM) and hematopoietic stem cell (HSC) transplantation as routine therapies for oncologic, hematologic, and immunologic disorders. Stem cells can be broadly divided into two categories: pluripotent stem cells (PSCs) and adult (somatic) stem cells. PSCs include embryonic stem cells and induced pluripotent stem cells, which have unlimited renewal capabilities and expansive repertoire of differentiation potential[9,10]. Adult stem cells include HSCs, endothelial progenitor cells, human amnion epithelial cells and mesenchymal stem/stromal cells (MSCs), which have a more limited renewal and differentiation potential. To date this vast array of stem cell types have had limited clinical use with the notable exception of HSCs and most of the therapeutic approaches have been focused on MSCs.

MSCs were first described in the 1970s as fibroblasts derived from the BM with colony-forming capabilities[11,12]. MSCs have been shown to differentiate into a variety of mesoderm-derived cells such as adipocytes, osteocytes, chondrocytes, fibroblasts, and skeletal muscle cells. MSCs can be derived from a variety of tissues including BM, adipose tissue, amniotic fluid (AF), umbilical cord (UC) Wharton's jelly, umbilical cord blood (UCB) and placenta. The ease of access, isolation, expansion, and *in vitro* differentiation potential of MSCs as well as their immunoprivileged nature have made them a popular stem cell choice in preventive and regenerative preclinical studies of human disease. Considering the heightened research interest in MSCs, the International Society for Cellular Therapy (ISCT) provided a minimal criteria for defining human MSCs, including (1) tissue culture plastic adherence, (2) expression of CD105, CD73 and CD90, (3) lacking expression of CD45 (pan-leukocyte), CD34 (hematopoietic and endothelial cells), CD14 or CD11b (monocytes and macrophages), CD79α or CD19 (B cells) and HLA-DR surface molecules, and (4) ability to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*[13]. This definition was updated in 2019 to recommend (1) including the tissue origin of cells, (2) use of stromal cell nomenclature unless rigorous evidence for stemness is shown and (3) including functional assays to define therapeutic mechanism of action, but no tissue- specific guidelines were addressed[14].

Transplantation of different adult stem cell types (predominantly MSCs) in preclinical models of neonatal disease has produced very promising results including immunomodulatory, anti-inflammatory, neuroprotective, angiogenic and regenerative effects[15-34]. Researchers initially assumed that MSCs exerted their therapeutic effects by homing to damaged tissues and differentiating into resident cells, however studies uncovered that the transplanted MSC cells neither underwent long-term engraftment nor differentiation[35-37]. Instead, the therapeutic action of MSCs has been determined to be mainly through paracrine mechanisms[15,38-41]. A multitude of studies have demonstrated that MSCs secrete a broad spectrum of bioactive compounds into the extracellular fluid and subsequently the bloodstream including growth factors, cytokines as well as EVs[36,42]. Multiple groups of investigators have gone on to prove that EVs are the key component that mediates the therapeutic effects of MSCs in numerous preclinical models of neonatal disease (Figure 1)[40,43-45]. EVs are a heterogeneous class of lipid-bilayer enclosed microparticles containing bioactive cargo without a nucleus that can be generated by almost all cell types. EVs were initially thought to be part of the cellular "garbage disposal" mechanism, through which corrupted or unwanted macromolecules are packaged and ejected from the cell. However, this mechanism has apparently evolved to also produce EV subpopulations designed for broadcasting signals and representing vectors of intercellular communication, arguably occasionally induced by environmental triggers [46-48]. The bioactive cargo of each EV subpopulation is highly variable and dependent on the type and state of the parent cells and their biogenetic pathway. EV cargo can be composed of any combination of lipids, proteins (enzymes, growth factors, receptors and cytokines), nucleotides (DNA, messenger RNA, long non-coding RNA and microRNA) and metabolites[46,49].

The mechanisms by which EVs mediate cell-cell signaling are still in the process of being discovered. Studies have shown that EVs can act on target cells by fusing with the target cell membrane to enter the cell and release the vesicle contents, interacting

with the receptor-ligand and inducing target cells to ingest EVs via endocytosis. Upon absorption from the environment, the EVs' cargo has the potential to impact a multitude of cellular processes including gene transcription, antigen presentation, cell proliferation, differentiation or apoptosis, and many others[50,51]. Even though the full definition of EV sub-types and their biogenesis are still being elucidated, in general EVs can be classified based on their size into 3 main types: (1) small EVs (including exosomes), defined as vesicles of approximately 30-150 nm in diameter and secreted through the endosomal pathway, (2) microvesicles, defined as vesicles of approximately 150-1000 nm in diameter that bud directly from the plasma membrane and usually have a membrane composition similar to that of the cell membrane (3) apoptotic bodies or blebs, defined as vesicles greater than 1 μm in diameter which are released by cells undergoing apoptosis[46-49]. For the remainder of this review, the term EVs will encompass all classes of EVs as endorsed by the International Society for Extracellular Vesicles (ISEV)[47]. There are several advantages of the stem cell secretome (such as EVs) over cellular approaches in clinical applications for the treatment of neonatal disease. First is the inherent nature of EVs being biologically inert and therefore less impacted by the environment. EVs also have a decreased risk of immunogenicity (such as rejection, tumor formation or autoimmune responses) and toxicity. Lastly, EV production, sterilization and storage are less complex compared with cell therapy[49]. Ultimately, all these EV-specific features are thought to facilitate the generation of a robust, ready-to-use, clinical-grade product for use in humans.

Bronchopulmonary Dysplasia

BPD is the most common respiratory complication of prematurity that affects 42% of very low birth weight (VLBW) infants in the United States[2]. Over the past few decades with the increasing survival of VLBW infants due to the use of antenatal steroids, surfactant supplementation and advancements of neonatal care, BPD remains the only complication of prematurity that continues to rise in prevalence[3]. BPD is the result of a complex multifactorial injury process in premature lungs that occurs in multiple stages and impacts lung development. Preterm birth disrupts late lung development and infants are born with lungs in the saccular stage of development prior to distal airway formation (alveolarization). In addition, both antenatal and postnatal insults can cause inflammation that impairs lung repair and can result in long-term remodeling in the lungs[52]. BPD is the most common chronic disease in children and can result in long-term complications such as increased risk of respiratory illnesses (viral infections, asthma, and emphysema), growth and neurodevelopmental impairment[53-58]. Despite progress in perinatal care and non-invasive respiratory management, the therapeutic options for BPD remain mostly supportive and minimally efficacious. In recent years, the stem cell secretome has emerged as a promising field of investigation for clinical application in BPD given its anti-inflammatory, immunomodulatory and angiogenic effects (Figure 1).

The interest in stem cell therapy originated from encouraging initial studies that showed beneficial effects of MSC therapy in pre-clinical models of BPD, which included improved survival, inhibition of inflammation, and attenuation in alveolar and lung vascular injury [15-17]. Aslam et al., from our group first revealed that cell-free conditioned media (CM) provided better protection than MSCs in a neonatal hyperoxia murine model of BPD,

which was subsequently confirmed by other groups[15,38,59-62]. Lee et al. then went on to demonstrate that MSC-CM mediated its therapeutic effects via a paracrine mechanism by identifying exosomes as the vehicle of protection in a model of hypoxia induced pulmonary hypertension, a known complication of severe BPD[43]. Subsequently, in Willis et al., our group were the first to demonstrate that a single dose of human UC derived-MSC-EVs (hUC-MSC-EVs) promoted alveolarization, decreased lung fibrosis, ameliorated pulmonary vascular remodeling, improved pulmonary function and alleviated associated pulmonary hypertension in the neonatal hyperoxia model[39]. Numerous studies from other groups have reproduced similar findings of MSC-EV's protective effects, including improved alveolarization, lung function, lung vascularization and pulmonary hypertension, using a variety of routes of administration in different models of BPD[63-71]. Our group has also gone on to show that MSC-EV treatment was not only capable of preventing hyperoxic lung injury but also contribute to partial reversal of established injury upon serial administration [72].

In terms of the mechanism of action of MSC-EVs, different hypotheses have been proposed with a core theme surrounding anti-inflammatory and immunomodulatory pathways. Willis et al. first proposed immunomodulation as the mechanism of action whereby MSC-EVs suppressed the pro-inflammatory "M1-like" macrophage state and enhanced the anti-inflammatory "M2-like" state[39]. Further studies by our group revealed that MSC-EVs localized to the lung, interacted with pulmonary myeloid cells, and restored alveolar macrophages in the injured lung. The interaction between MSC-EVs and myeloid cells promoted an immunosuppressive, $Ly6C^+$ (lymphocyte antigen 6 complex, locus C1)/ LyG^+ (lymphocyte antigen 6 complex, locus G), $CX3CR1^+$ (C-X3-C motif chemokine receptor 1) and $CCR2^-$ (C-C motif chemokine receptor 2) myeloid cell phenotype. Additionally, transplantation of MSC-EV-educated BM-derived myeloid cells paralleled the therapeutic effects as MSC-EV treatment. Functional studies helped elucidate that MSC-EVs induced transcriptomic and epigenetic reprogramming of the monocytes via modulation of the CCR2/CCL2 (C-C motif chemokine ligand 2) axis, which is involved in the infiltration and recruitment of monocyte-derived suppressive cells with high levels of anti-inflammatory activities such as Arginase 1 and interleukin 10 (IL-10)[73].

Similarly, Chaubey et al. proposed that TSG-6 (tumor necrosis factor-stimulated gene-6), an immunomodulatory glycoprotein detected in MSC-EVs was responsible for their mechanism of action. TSG-6 induced a macrophage phenotypic shift from "M1-like" towards "M2-like" profile[64]. The group showed that administration of TSG-6 *in vivo* attenuated BPD and its associated pathologies in the lung (alveolar-capillary leakage and alveolar simplification), heart (pulmonary hypertension and right ventricular hypertrophy) and brain (neuronal apoptosis and hypomyelination). They also demonstrated that knocking down TSG-6 in MSC-EVs abolished their therapeutic effects almost entirely[64]. Porzionato et al. also demonstrated that hyperoxia exposure reduced "M2-like" macrophages in interstitial, alveolar and perivascular populations, which were prevented by hUC-MSC-EV treatment and suggestive that M2 macrophage polarization could play a role in MSC-EVs' anti-inflammatory effects[68]. Lithopoulos et al. also demonstrated the immunomodulatory capabilities of hUC-MSC-EVs in a multifactorial neonatal lung injury model induced by endotoxin and ventilation whereby treatment resulted in decreased expression of pro-

inflammatory marker IP-10 (interferon-γ induced protein 10) and increased expression of anti-inflammatory markers (IL-4, IL-13) in the lung[71].

Other groups have been proponents of a different MSC-EV mechanism of action, namely its pro-angiogenic and pulmonary vascularization effects mediated via VEGF (vascular endothelial growth factor)[63,65,67]. Ahn et al. first demonstrated this by knocking down VEGF in hUC-MSC-EVs, which impaired their therapeutic effects in neonatal hyperoxic lung injury both *in vitro* and *in vivo*. Interestingly, they also showed that the MSC-EVs were internalized by vascular pericytes, macrophages, type 2 epithelial cells and fibroblasts but not by endothelial cells, which suggests paracrine crosstalk between these cells mediating the pro-angiogenic effects of MSC-EVs[63]. Braun et al. also showed that murine BM derived-MSC-EVs (mBM-MSC-EVs) had VEGF-dependent pro-angiogenic effects using an *in vitro* human umbilical vein endothelial cell tube formation assay to demonstrate increased angiogenic capabilities, which were reversed with anti-VEGF antibody treatments. They also used *in vivo* rodent hyperoxia model to show that MSC-EVs improved alveolar growth, increased small blood vessel number and size as well as inhibited right ventricular hypertrophy[65]. You et al. demonstrated similar findings of hUC-MSC-EVs improving alveolarization and angiogenesis by protecting type II alveolar epithelial cells and pulmonary vascular endothelial cells via the PTEN (phosphatase and tensin homolog)/Akt (protein kinase B) signaling pathway[67].

MSC-EVs are known to carry a variety of bioactive cargo, which can include microRNAs (miRNAs). miRNAs are small non-coding RNAs that modulate gene expression on the post-transcriptional level. Some investigators have postulated that miRNAs may be a potential mechanism of action for MSC-EVs. Hyperoxia exposure in rodents results in a reduction of miR21-5p[74]. Wu et al. showed that murine adipose tissue-derived MSC-EVs attenuated the effects of hyperoxia in neonatal mice by transferring miR-21-5p into lung cells, which resulted in modulation of C/EBPα (CCAAT-enhancer-binding protein α) expression, a key transcription factor in perinatal lung maturation and lung epithelial repair[75,76]. The same group has also shown that miR-425 in rodent BM-MSC-EVs (rBM-MSC-EVs) attenuated hyperoxic injury by targeting PTEN and activating the PI3K (phosphatidylinositol-3-kinase)/Akt axis[69]. Other proposed mechanisms of action of MSC-EVs include differentiation of lung cell types and protein regulation. Ai et al. demonstrated that hUC-MSC-EVs suppressed transdifferentiation of alveolar type 2 cells into type 1 cells (which is increased in hyperoxic neonatal lung injury) through downregulation of Wnt5 (Wingless/Integrase 1 family member 5)[70,77]. Li et al. reported that human amnion derived-MSC-EVs ameliorated neonatal hyperoxic lung injury through increased APEH (acylaminoacyl-peptide hydrolase) expression, which is a proteasome that facilitates clearance of oxidatively damaged proteins as part of the cellular response to DNA damage[78].

Hyperoxia exposure also has multi-organ effects beyond the cardio-pulmonary system including the thymus, brain, and eye (which will be discussed in a separate section). From our group, Reis et al. reported that hyperoxia induced involution of the thymic medulla, disruption of $FoxP3^+$ (forkhead box P3) T regulatory cells and increased T cell autoreactivity, all of which suggests disruption of central tolerance and adaptive immunity[79]. hUC-MSC-EV treatment restored thymic architecture and functionality via

the thymic medullary antigen presentation axis with enrichment of antigen presentation and anti-oxidative-stress related genes in dendritic cells and medullary thymic epithelial cells[79]. Fernandez-Gonzalez et al. demonstrated that hyperoxia resulted in multiorgan effects in the brain and retina. Regarding the neurologic impacts of hyperoxia, the group demonstrated decreased myelination and increased astrogliosis, which induced activation of microglial cells in the brain, all of which was prevented by a single dose of hUC-MSC-EVs[80]. Lithopoulos similarly used a multifactorial neonatal mouse model of lung injury to demonstrate neurologic impacts with impaired neural progenitor cell function, which was mitigated by hUC-MSC-EVs[71].

To date, there have been several published clinical trials of stem cell therapy in BPD including different MSC sources such as the UC and amnion. Chang et al. were the first group to publish a Phase I dose-escalation trial of allogeneic hUC-MSCs administered intratracheally in 9 extremely preterm infants (<30 weeks gestation) at risk for BPD. They showed that MSC treatment was well-tolerated with no serious adverse effects and that it resulted in a reduction of pro-inflammatory cytokines IL-6 and tumor necrosis factor-α (TNF-α) in tracheal aspirates and reduced BPD severity[81]. They also demonstrated long-term safety (growth, respiratory and neurodevelopment) with MSC administration in the same group of patients[82]. Powell et al. recapitulated similar findings of safety and feasibility in another Phase I dose-escalation trial of allogeneic hUC-MSCs in 12 extremely low birth weight (<28 weeks gestation and < 1000 g) infants [83]. Ahn et al. conducted the first Phase II double-blind, randomized, placebo-controlled trial of hUC-MSCs in 33 extremely preterm infants (<29 weeks gestation) receiving mechanical ventilation with respiratory deterioration between postnatal days 5-14. There was no statistical significance in the primary outcome of death or moderate-severe BPD however there was a significant reduction in severe BPD (19% in treatment group versus 53% in control group) in the most extremely preterm infants at 23-24 weeks in the subgroup analysis[84]. The same group is currently conducting an additional larger and controlled phase II clinical trial focused on infants between 23-24 weeks gestation (NCT003392467).

Ren et al. performed a Phase I placebo-control trial of autologous umbilical cord mononuclear cells in 15 preterm infants (< 35 weeks gestation) and showed no difference in mortality or preterm complication rates however there was a reduced duration of mechanical ventilation and oxygen therapy[85]. Lim et al. conducted a Phase I trial of allogeneic human amnion epithelial cells (which are stem-like cells derived from placental membranes) in 6 preterm infants with BPD, that showed safety of the IV infusion and no adverse effects[86]. The two-year outcome data similarly showed no evidence of long-term adverse effects[87]. To our knowledge, there has only been one clinical trial utilizing the stem cell secretome, a Phase I study to evaluate the safety of MSC-EVs in BPD, which was discontinued due to business decisions (NCT03857841). The clinical trials listed above have promising safety and feasibility results in small cohorts of preterm infants and preliminary signs of efficacy in the extremely preterm infants, which is encouraging for the field and at present there are 8 ongoing trials of stem cell therapy in BPD[88].

Hypoxic-Ischemic Encephalopathy & Perinatal Arterial Ischemic Stroke

HIE is a form of perinatal brain injury that occurs due to oxygen deprivation to the brain that can result in neonatal encephalopathy. The incidence of HIE is estimated to be 1-3 per 1000 live term births in developed countries and accounts for 15-35% of all cases of neonatal encephalopathy[89,90]. HIE is a significant cause of mortality and neurologic morbidity including neurodevelopment disability, neurosensory impairment, epilepsy, and cerebral palsy (CP) in term infants. HIE may occur by one or more of the following mechanisms: impaired oxygenation or inadequate perfusion of the maternal placenta, placental pathology and impaired fetal oxygenation or perfusion[91]. HIE injury occurs through 3 phases including the initial hypoxic-ischemic insult, followed by secondary effects, such as mitochondrial deficiency, oxidative stress, excitotoxicity inflammation and early stages of neuronal necrosis and apoptosis and the third phase, which encompasses long-term cell death, inflammation, cell turnover and repair and gliosis[92,93]. Currently the only therapy with proven efficacy for HIE is therapeutic hypothermia (TH), which is hypothesized to reduce the negative consequences of secondary injury and inflammation[94,95]. Multiple TH trials have demonstrated improved outcomes (survival, neurodevelopmental disability, CP, and neurosensory deficits) but it does not completely alleviate the effects of HIE[94,96-99]. In addition, TH must be initiated promptly (within 6 hours of life) to be beneficial. Researchers continue to search for other agents and strategies to improve HIE treatment and outcomes and one of the encouraging pursuits is stem cell therapy.

There have been numerous pre-clinical studies of hypoxic brain injury demonstrating that MSC therapy results in significant neuroprotection, increased neuro-regeneration, attenuated neuroinflammation and improved functional outcomes[18-20,100]. Investigators have also evaluated strategies to augment the therapeutic capabilities of MSCs including thrombin preconditioning and genetic modification[101-103]. Other groups have combined MSC therapy with standard of care treatment, TH and found that the combination was capable of augmenting neuroprotective activity and lengthening the therapeutic time window[104,105]. Recently there has been interest in evaluating other sources of stem cells. Huang et al. demonstrated that human PSC-derived ectomesenchymal stromal cells were capable of inducing more functional recovery in hypoxic-ischemic (HI) brain damage than hUC-MSCs[106]. With regards to the stem cell secretome, there have been multiple studies demonstrating similar beneficial effects to MSCs (as listed above)[40,107-115]. Wei et al. published the first study to suggest therapeutic effects of the stem cell secretome by demonstrating that CM from adipose-derived MSCs conferred neuroprotection in a rodent model of HIE[107]. Ophelders et al. went on to become the first group to demonstrate that human BM MSC-EVs (hBM-MSC-EVs) could provide neuroprotection by improving brain function, reducing total number and duration of seizures and restoring subcortical white matter myelination in an ovine model of HIE[40]. Interestingly, they reported that cerebral inflammation was unaffected by MSC-EV administration, but subsequent studies from independent groups have demonstrated MSC-EV therapy has an anti-inflammatory effect on the brain[108,109,111-113,115].

One of the main hypotheses proposed regarding the mechanism of action of the MSC-EVs is their anti-inflammatory effects modulated by the MAPK (mitogen-activated protein

kinase) and NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling pathway. Kaminski et al. showed that hBM-MSC-EVs resulted in anti-inflammatory effects via significant downregulation of pro-inflammatory cytokine, TNFα and upregulation of anti-inflammatory cytokine, TGFβ (transforming growth factor β)[109]. In contrast, Drommelschmidt showed that hBM-MSC-EVs ameliorated inflammation-induced cellular damage but did not affect systemic and cerebral pro-inflammatory cytokine expression[108]. Thomi et al. proposed that hUC-MSC-EVs reduced microglia mediated neuroinflammation by interfering with TLR-4 (Toll-like receptor 4) signaling pathway and preventing degradation of NFκB inhibitor, IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha) and phosphorylation of the MAPK family[111]. Shu et al. demonstrated that hBM-MSC-EVs' anti-inflammatory effects on microglia were mediated by inhibition of P38MAPK/P65NFκB signaling pathway resulting in dampened transcription of inflammation-related genes[115].

There have also been multiple groups that have postulated that the neuroprotective effects of MSC-EVs are mediated via the delivery of miRNAs and subsequent immunomodulation. Joerger-Messerli et al. showed in an *in vitro* model of oxygen/glucose deprivation/reoxygenation (OGD/R) injury that hUC-MSC-EVs contained miRs let-7a and let-7e and released their contents into the neuronal cells[41]. Given that miRs let-7a and let-7e are known regulators of Casp3 (caspase 3), the group proposed that MSC-EV treatment inhibited Casp3 expression and subsequent HI induced apoptosis of neuronal cells[41]. Xin et al. used an *in vivo* murine model of HIE to demonstrate that mBM-MSC-EVs transferred miR-21a-5q to neurons and microglia therefore conferring neuroprotection via the Timp3 (tissue inhibitor of metalloproteinase 3) pathway[113]. In addition, they also showed that MSC-EV treatment had immunomodulatory effects by skewing both microglia and brain monocyte/macrophage towards an anti-inflammatory phenotype[113]. The same group also went on to show that delivery of miR-21a-5p by mBM-MSC-EVs induced microglial M2 polarization via the STAT3 (signal transducer and activator of transcription 3) pathway[114]. Chu et al. showed that hydrogen sulfide preconditioned mBM-MSC-EVs had increased therapeutic efficacy (neuroprotection, anti-inflammation and improved long-term cognitive and memory outcomes) and that MSC-EVs transferred miR-7b-5p into the ipsilateral cortex, which further induced its expression therefore promoting an anti-inflammatory microglia and mononuclear phagocyte phenotype[116]. Han et al. proved successful delivery of miR-410 by hUC-MSC-EVs to neurons which inhibited neuronal apoptosis via histone deacetylase dependent EGR2 (early growth response 2)/Bcl2 (B-cell lymphoma 2) axis[117].

Other proposed mechanisms of action of MSC-EVs' neuroprotection in HI induced brain injury include strengthening of the blood-brain-barrier (BBB), enhancing mitophagy and increasing growth factor expression. Gussenhoven et al. demonstrated that hBM-MSC-EVs contained Annexin A1 (ANXA1) and that both MSC-EVs and human recombinant ANXA1 could improve the integrity of the BBB via the formyl peptide receptor pathway in both a fetal ovine HIE model and an *in vitro* OGD/R injury model[118]. Hu et. al used the same OGD/R model to prove that hUC-MSC-EVs protected microglia from ischemia/reperfusion injury by enhancing mitophagy via an upregulation of FOXO3a (Forkhead box O3a) expression[119]. Kaminski et al. has demonstrated that hBM-MSC-EVs lead to an increase in neuronal growth factor expression, which mediates neuro-regenerative responses[109].

At present, there have been three Phase I trials demonstrating safety and feasibility of autologous UCB cells for neonates with HIE[120-122]. Studies from Cotten et al., suggested potential efficacy with 74% of recipients of cell infusion versus 41% of cooled controls obtaining normal neurodevelopmental scores in 3 domains of the Bayley-III, however this finding did not reach statistical significance[121]. There have also been two Phase I trials revealing the feasibility of allogeneic UCB-derived MSCs[123,124]. Currently there are 9 clinical trials of stem cell therapy in neonatal HIE[88]. With regards to clinical trials of stem cell secretome in HIE, thus far there has only been one study exploring the safety and efficacy of MSC's paracrine effects and unfortunately the status of study is unknown (NCT02854579).

The MSC secretome has also been studied in other pre-clinical models of perinatal brain injury such as arterial ischemic stroke (AIS) and brain injuries related to prematurity, which will be discussed in the following section. Perinatal AIS is an occlusive cerebral arterial event, typically thromboembolic in nature however the exact pathogenesis remains unknown and unlike adult stroke, there are minimal interventions available for perinatal AIS. There have been a few studies that have revealed beneficial effects of MSC therapy in pre-clinical models of perinatal AIS[125-128]. Erythropoietin enhanced rodent adipose tissue-derived MSC improved long-term neurobehavioral outcomes in an *in vivo* rodent model of neonatal stroke[129]. Pathipathi et al. used the same model to show that mBM-MSC-EVs accumulated in activated microglia and macrophage in the injury site and attenuated a subset of cytokines and chemokines induced by transient ischemia, which may contribute to a reduction in Casp3-dependent apoptosis[130]. Recently, the first clinical trial of intranasal hBM-MSCs in neonates with perinatal AIS stroke was published, which demonstrated both feasibility and safety[131].

Intraventricular Hemorrhage and Periventricular Leukomalacia

IVH is the most common neurologic complication of prematurity and estimated to occur in 25-30% of VLBW infants[132]. IVH is an important cause of morbidity as it places infants at risk for adverse neurodevelopment outcomes, which increases with IVH severity[2,133,134]. The pathophysiology of IVH relates to the inherent germinal matrix fragility in premature infants and the interplay with disturbances in cerebral blood flow (CBF)[135-137]. This is further compounded by the impaired cerebral vascular autoregulation in the immature brain, which leads to an inability to maintain constant CBF during changes in systemic pressure[138]. The clinical presentation of IVH is often silent and typically only detected on screening cranial ultrasounds. Prevention of brain injury in preterm infants has been predominantly focused on maintaining constant CBF and avoiding any fluctuations, which can be challenging given the lack of autoregulation and lack of CBF measurement technology. Currently there is only supportive therapy available for IVH with no active therapies or strategies to prevent the development of its complications. The incidence of severe IVH has changed minimally in the past two decades despite neonatal advancements therefore new therapies are needed to help mitigate morbidity[2].

After the development of IVH, the most common complications that arise are posthemorrhagic hydrocephalus (PHH) and PVL. There are interventions available for

progressive and symptomatic PHH that focus on cerebrospinal fluid drainage such as serial lumbar punctures, ventricular access devices or more permanent ventricular shunts. PVL consists of periventricular focal necrosis with subsequent cystic formation and more diffuse cerebral white matter injury[139]. PVL is a result of secondary brain injury induced by IVH and the exact mechanism remains unknown however there are several factors involved including microglia and astrocyte activation, degradation of blood components with release of “toxic” products, infiltration of the brain by immune cells, death of neuronal and glial cells and arrest of preoligodendrocyte maturation, which ultimately results in BBB dysfunction[140]. PVL injury is also often silent in presentation and can best be detected on magnetic resonance imaging whereby its pattern of injury can be described as either cystic or diffuse[139]. Cystic PVL tends to damage deeper, more medial tracts that control lower extremity motor function, resulting in spastic diplegia whereas injury to the more lateral tracts may cause upper extremity impairment and extensive white matter injury may result in quadriplegia. On the other hand, diffuse PVL is the most common form of brain injury and the major cause of cognitive deficits and chronic development impairment in preterm infants[141]. Unfortunately, there is no treatment available to prevent or treat PVL and current standard of care is symptomatic management and rehabilitation.

There have been multiple studies reporting that MSC therapy attenuated further cortical injuries and progressive hydrocephalus in a rodent neonate model of severe IVH induced by intracerebroventricular (IC) injection of blood[22-27]. Ahn et al. demonstrated that human UCB-MSC (hUCB-MSC) treatment significantly attenuated impairment on behavioral tests, sensorimotor coordination and neuronal loss, as well as promoted neurogenesis in the hippocampus[24,27,142]. The group also proposed a paracrine mechanism of action of MSCs and identified brain-derived neurotropic factor (BDNF) as a factor secreted by hUCB-MSCs that mediated its neuroprotective effects via the BDNF/TrkB (tyrosine kinase B)/CREB (cAMP response element-binding protein) signaling axis activation[23,27]. The same group also conducted numerous optimization studies of hUCB-MSC therapy in IVH and found that MSCs conferred neuroprotection only with early administration (2 days after induction of IVH) and that both IC and IV routes had equivalent therapeutic efficacy in protecting against severe IVH[22,25]. More recently, they have also demonstrated that thrombin preconditioning of hUCB-MSCs significantly improved their therapeutic efficacy compared to naïve MSCs and was more effective at reducing brain injury including progressive PHH, astrogliosis, neuronal cell death, inflammation, and neurobehavioral impairment[143]. Moreover, the first study evaluating the application of stem cell secretome in IVH was published by the same group whereby they found that a single IC injection of hUCB-MSC-EVs conveyed equivalent neuroprotection to MSC therapy via BDNF signaling[44].

At present, there have been a few clinical trials investigating the safety and feasibility of stem cell therapy in IVH and its complications but no trials of the secretome yet. Ahn et al. conducted a Phase I dose escalation clinical trial of allogeneic UCB-MSC therapy in 9 extremely preterm infants with severe IVH, which showed that the IC injection procedure was well-tolerated with no significant adverse effects reported by term-corrected age[144]. Sun et al. performed a phase I trial of autologous UCB for congenital hydrocephalus, which showed that repeated IV infusions of UCB were both safe and feasible[145]. Currently, there are two ongoing clinical trials for IVH: first is a Phase II randomized controlled trial of

UC-MSCs assessing efficacy, safety and feasibility in preterm infants with severe IVH that is in the active stage of recruiting (NCT02890953), second is a Phase I trial of autologous UCB cells in extremely preterm infants (< 28 weeks gestation prior to development of IVH) to evaluate for safety and feasibility with the eventual goal to assess for neuroprotection (ACTRN12619001637134)[146].

There have also been some promising studies of MSCs and their secretome utilized as therapy in pre-clinical models of PVL but no reports of any clinical trials yet. Zhu et al. demonstrated that a single intraperitoneal injection of hUC-MSCs increased mature oligodendrocytes, decreased reactive astrocytes and activated microglia cells and markedly improved functional outcomes (sensorimotor and cognitive function) in a rodent model of PVL[28]. Oppliger et al. used an *in vitro* model to demonstrate that hUC-MSCs caused increased oligodendrocyte differentiation in neural progenitor cells (NPCs) via cell-to-cell contact and that trophic factors secreted in CM were capable of promoting astrogenesis. They also found that term hUC-MSCs caused a stronger oligodendroglial differentiation effect in NPCs than pre-term hUC-MSCs and proteomic analysis of CM identified the laminin-α2-subunit as a potential factor for the difference[29]. Morioka et al. showed that CM from interferon γ (IFNγ) preconditioned hUC-MSCs decreased pro-inflammatory cytokines in the brain and improved myelination in a rodent model of PVL induced by lipopolysaccharide (LPS) injection[30].

Necrotizing Enterocolitis

NEC is a devastating neonatal gastrointestinal disease that occurs in 1-3 per 1000 live births every year in the United States with high rates of morbidity and mortality[3]. NEC is characterized by extensive intestinal inflammation, ranging from mucosal injury to full thickness necrosis and perforation that often leads to systemic inflammation affecting distal organs[147-149]. The etiology of NEC is multifactorial with numerous risk factors that have been identified, the most widely recognized are low gestational age, low birth weight and formula feeding. The pathogenesis of NEC stems from the susceptibility and hyperreactivity of the preterm gut, which predisposes the intestine to altered immune response, vascular injury, and dysregulated microbiome[150]. The clinical presentation of NEC ranges widely from subtle non-specific symptoms to severe hemodynamic instability. Diagnosis of NEC is typically made with abdominal radiographic findings such as gas filled bowel loops, pneumatosis intestinalis, portal venous gas or pneumoperitoneum. NEC prevention includes cautious advancement of enteral feedings and the exclusive use of breastmilk in high-risk patient populations. The mainstay of NEC treatment includes bowel rest by withholding enteral feedings, gastric decompression, broad spectrum antibiotics and in severe cases, surgical resection. Despite medical and surgical advances, mortality of NEC remains high between 20-40% with younger gestational age infants and infants requiring NEC surgery demonstrating higher mortality[151]. There are also long-term sequelae of NEC including short gut syndrome, intestinal stricture, intestinal failure and neurodevelopmental delays[152]. Researchers have been exploring different strategies for NEC treatment such as stem cell therapy.

There have been various studies from different groups demonstrating the efficacy of MSC therapy in preclinical rodent models of NEC. Primary effects of MSC therapy reported include improved survival and weight gain, decreased mucosal damage and improved gut barrier function[78,153-159]. Interestingly, there have been two separate reports of AF-MSCs conferring protective effects in NEC but not BM-MSCs[155,160]. There have been different mechanisms proposed regarding the mechanism of action of MSC therapy in NEC including different signaling pathways such as COX-2 (cyclooxygenase 2), Wnt and NFκB-IGF-1 (insulin growth factor 1)-TGFβ[78,155,159]. Drucker et al. demonstrated that hUC-MSCs may exert their beneficial effects in NEC via the production of paracrine mediator hydrogen sulfide at low levels, which conferred cytoprotective, antioxidant and anti-inflammatory functions[158]. The myenteric plexus ganglia are damaged in NEC patients and neural stem cells (NSCs) are responsible for repairing and renewing neurons in the enteric nervous system hence there has been an interest in investigating their therapeutic effects[161]. Investigators have utilized both *in vivo* and *in vitro* models of NEC and found NSC therapy to be effective in decreasing NEC incidence and mortality as well as improving the enteric nervous system, gut barrier function and intestinal motility[162-164]. One group demonstrated that heparin-binding epidermal growth factor-like factor promoted NSC proliferation and enhanced nitric oxide production via increasing expression of neuronal nitric oxide synthase, which conferred protection on the neurons from degeneration and damage[163,164]. Considering the future clinical applications of this therapy, collecting NSCs from fetal sources is not feasible hence AF has been studied as an alternative source of NSCs. McCulloh et al. used a rodent model of NEC to directly compare different sources for stem cells including BM-MSC, AF-MSC, AF-NSC and enteric NSC (E-NSC). They found that all stem cell treatments resulted in a significant decrease in intestinal permeability and increase in gut barrier function with no difference between the different types[157,165].

Rager et al. was the first group to demonstrate therapeutic efficacy of stem cell derived EVs and its equivalence to stem cell therapy in NEC. They found that rBM-MSC-EVs increased wound healing *in vitro* and significantly decreased gut permeability and incidence of NEC *in vivo*[166]. McCulloh et al. performed a similar comparison study between different rodent sources of EVs including BM-MSC, AF-MSC, AF-NSC and E-NSC and found that all four demonstrated equivalent reductions in NEC incidence and that EVs were equivalent to direct stem cell therapy as well[167]. Li et al. also demonstrated in a murine model of NEC that rodent AF-MSC-EVs increased cellular proliferation, reduced inflammation and regenerated normal intestinal epithelium via increasing intestinal stem cell activity and Wnt pathway activation, which ultimately reduced the incidence of NEC. The group also showed that EVs failed to prevent injury if administered prior to NEC induction highlighting the importance of timing of therapy[78]. Majority of the studies to date utilized rodent sources of stem cells, more recently the same group showed that both CM and EVs from human AF-MSC (hAF-MSC) were also effective in reducing NEC incidence, intestinal inflammation and injury while increasing intestinal stem cell activity[168,169]. Proteomic analysis of the hAF-MSC-CM revealed several protein clusters including immune-regulation, cell cycle and stem cell regulation, which may further elucidate their mechanism of action[169]. Thus far, there has yet to be any clinical trials of stem cell or secretome therapy in NEC and only one case report of a 22-day old patient with NEC who underwent a laparotomy and

had 60 cm of necrotic bowel resected, and subsequently received IV allogeneic UC-MSCs who demonstrated improvement in intestinal blood supply and did not develop short bowel syndrome[170].

Retinopathy of Prematurity

ROP is a vision-threatening disease characterized by abnormal retinal vascular development that predominantly affects premature infants and is the leading cause of avoidable childhood blindness worldwide[171,172]. ROP is also a lifetime disease with deleterious impacts on other ocular diseases such as glaucoma, amblyopia, strabismus, myopia and retinal detachment[173].The two major factors in the pathogenesis of ROP are the immaturity of retinal vessels and oxygen exposure resulting in oxidative injury[174]. ROP progression occurs in two phases: in phase 1 there is cessation of normal retinal vascular development due to a loss of growth factors (IGF-1 and VEGF) secondary to hyperoxia and loss of the maternal-fetal interface however the retina continues to mature with increasing metabolic demands therefore resulting in relative hypoxia, and in phase 2 the hypoxic retina stimulates the expression of oxygen-regulated factors such as erythropoietin and VEGF, which induce retinal neovascularization resulting in disorganized and leaky retinal vessels, predisposing to retinal detachment[174,175]. ROP prevention strategies include limiting oxygen exposure and toxicity by establishing oxygen saturation target parameters and routine ROP screening examinations to monitor for disease development and progression[176]. Current therapy options for ROP include laser photocoagulation, anti-VEGF agents and in severe cases, scleral buckling and/or vitrectomy. Laser photocoagulation is the standard of treatment for threshold (condition with 50% risk of retinal detachment) or severe ROP (partial or complete retinal detachment), whereby the non-vascularized retina is ablated to prevent further inflammation and detachment[177]. Laser therapy is a lengthy procedure, which requires sedation or general anesthesia and can result in serious vision-threatening complications[177]. Anti-VEGF therapies are a recent development in the treatment of threshold ROP either as a single treatment or as an adjunct to laser therapy. Inhibiting VEGF prevents abnormal vasoproliferation and facilitates physiologic retinal vascular development. Anti-VEGF therapy appears to be as effective as laser therapy however at present there is a lack of long-term outcome and safety data[178]. Anti-VEGF agents are administered by intravitreal injection, which is an invasive procedure with the risk of vision-threatening complications hence there is a need for less invasive and potentially curative therapies for ROP [178].

There have been several groups that have shown promising results of MSC therapy in pre-clinical ROP models such as retinal ischemia/reperfusion injury and oxygen-induced retinopathy (OIR). MSC treatment was able to reduce retinal neovascularization, preserve retinal thickness and prevent the loss of retinal ganglion cells[31,33,34,179]. In terms of the therapeutic effects of the MSC secretome, Moisseiev et al showed that hBM-MSC-EVs partially preserved retinal vascular flow, reduced retinal thinning and retinal neovascularization in an *in vivo* OIR model. Proteomic analysis of MSC-EVs demonstrated the presence of pro-survival associated proteins from the CREB pathway. They also demonstrated that MSC-EV treatment did not induce any immunogenicity or adverse effects[45]. Mathew et al. demonstrated that hBM-MSC-EVs attenuated cell death in an *in vitro* OGD model. They also showed significantly improved functional recovery, decreased

neuro-inflammation and apoptosis in an *in vivo* rodent model of retinal ischemia[180]. Noueihed et al showed that both mBM-MSCs and their CM could inhibit neovascularization and diminish vasoobliteration in the OIR model by restoring neural Sema3E (semaphorin 3E) levels, which in turn reduced levels of IL17A and -other pro-inflammatory factors[34]. More recently Fernandez-Gonzalez et al., demonstrated that hyperoxia induced multi-organ damage including retinal thinning, induction of gliosis and microglial activation and invasion into the outer nuclear layer, which were all prevented by a single IV dose of hUC-MSC-EVs[80].

Placental Diseases: Preeclampsia and Chorioamnionitis

The placenta serves as the maternal-fetal interface essential for the delivery of nutrients and oxygen from mother to fetus to maintain normal fetal growth[181]. Placental diseases such as preeclampsia (PE) and chorioamnionitis can have significant impact on the well-being of the fetus during pregnancy such as fetal growth restriction (FGR), preterm birth and in severe cases, stillbirth. In addition, these placental diseases can also have longer-lasting effects on neonatal health that are just starting to be understood. Sequelae of placental diseases such as growth restriction and an inflammatory intrauterine environment are both known to have an adverse effect on the developing lung and are significant risk factors for the development of BPD. PE is a gestational-specific maternal systemic vasculopathy affecting 3-5% of all pregnancies[182]. The pathogenesis of PE is characterized by impaired spiral arteriole remodeling resulting in hypoperfusion of the placenta and an imbalance in pro- and anti-angiogenic factors, which when released in the maternal circulation alter systemic endothelial function resulting in the clinical manifestations of PE. PE presents most commonly with maternal symptoms of hypertension, proteinuria, and if left untreated liver dysfunction, cerebral edema, and seizures. Current PE treatment is focused on alleviating maternal preeclamptic symptoms but there are no current available therapies to prevent PE, its progression, or adverse fetal effects. Chorioamnionitis is an inflammatory disorder of the chorion, amnion or both that can be due to an intra-amniotic infection, sterile inflammation, environmental pollutants, cigarette smoke and other toxicants[183]. Chorioamnionitis is a frequent cause of preterm birth with 40-70% of cases attributed to it[184-186]. The inflammatory mediators released in chorioamnionitis are indicated as the causative agents of preterm labor and/or premature rupture of membranes. Chorioamnionitis is also associated with a multitude of serious neonatal complications including perinatal death, early-onset neonatal sepsis, septic shock, pneumonia, meningitis, IVH, cerebral white matter damage, ROP, NEC, respiratory distress syndrome and BPD[183]. Current treatments for chorioamnionitis and/or preterm labor include antibiotics, progesterone, and antenatal corticosteroids however none of these treatments ameliorate the neonatal morbidities.

Recently there has been an emerging interest in studying the effects of the MSC secretome in placental diseases. Xiong et al. showed that intra-abdominal administration of hUC-MSC-EVs on gestational day 14 for 6 days total was able to decrease blood pressure and urinary protein levels in a rodent model of PE induced by injections of a nitric oxide synthase inhibitor, NG-nitro-L-arginine methyl ester. Their group also illustrated that MSC-EVs were capable of improving placental quality and promoting angiogenesis[187]. Wang et al. used an *in vitro* model of human extravillous cytotrophoblast derived transformed cell lines to

demonstrate that hUC-MSC-EVs were capable of boosting trophoblast cell proliferation, migration and invasion via restoration of miRNA 133b, which subsequently restricted SGK1 (serum/glucocorticoid regulated kinase 1)[188]. Recently, our group led by Taglauer et al. has demonstrated that a single antenatal dose of hUC-MSC-EVs can prevent pregnancy loss, FGR and PE physiology in a preclinical *in vivo* model of PE (heme oxygenase null mouse). Mass cytometric analysis of utero-placental leukocytes demonstrated that antenatal MSC-EV treatment resulted in intrauterine immunomodulation impacting abundance, surface marker repertoire and cytokine profiles[189]. They also demonstrated that the newborn pups of preeclamptic mothers exhibited significant alveolar simplification, altered bronchial epithelial morphology and alterations in lung developmental genes, which is consistent with the clinical impact of PE on the developing lung. Weekly antenatal administration of MSC-EVs normalized fetal lung branching morphogenesis and prevented the prior deleterious lung effects by altering the expression of multiple inflammatory mediators in preeclamptic AF[190]. Abele et al. utilized a rat model of endotoxin-induced chorioamnionitis and demonstrated that it resulted in reduced alveolarization and pulmonary vessel density, increased right ventricular hypertrophy and decreased lung mechanics in rat pups, which are findings similar to BPD. They went on to show that intrauterine administration of hBM-MSC-EVs was able to reduce placental inflammatory gene signaling NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) and IL-1β , improve trophoblast cell invasion and normalize spiral artery architecture. Additionally, the pups treated with MSC-EVs demonstrated preserved distal lung growth and mechanics. Further *in vitro* studies of fetal lung explants showed that EV treatment had a direct effect on the lung by enhancing distal lung branching and restoring VEGF and SPC (surfactant protein C) gene expression[191].

Challenges to Therapeutic Applications of Stem Cell Secretome in Neonatal Diseases

The promising results of stem cell secretome therapy (mostly MSC-EVs) in preclinical models of neonatal disease has contributed to the growing interest in its clinical applications in patients. The majority of stem cell based clinical trials in neonates have focused on stem cell transplantation itself[81-85,121-124,131,144,145]. Currently there have only been 2 reported clinical trials of stem cell secretome therapy (NCT03857841, a safety study of MSC-EVs in severe BPD and NCT02854579, a study to evaluate the safety and efficacy of MSC's paracrine effects in HIE), which were both sadly unable to yield results for different reasons. The demonstrated safety and feasibility of stem cell therapy in both term and preterm neonates and the promising results from preclinical studies of MSV-EV therapy is highly suggestive of the clinical potential of the stem cell secretome. The field of stem cell secretome research is still in its early stages and there are various challenges ahead of its clinical applications, ranging from mechanistic to technical.

First off, further preclinical studies are required to identify the mechanism of action of EVs in each neonatal disease process. EVs are diverse molecules that carry a multitude of bioactive cargo including protein, lipids, genetic material, and metabolites, which can exert molecular and epigenetic effects on target cells that can be difficult to tease apart. Several studies mentioned in this review have identified different singular miRNAs or proteins as

the effector molecule of EV function, however given the complex effects of EVs and their vastly heterogeneous cargo this makes a single moiety being responsible for their therapeutic action highly unlikely. It is more likely that a combination of different active elements orchestrates their therapeutic efficacy. Given that EV cargo is highly dependent on its parent cell, there has been some consideration to exposing parent cells to varying culture conditions such as low pH or low oxygen concentrations to influence EV secretion and its subsequent cargo[192,193]. Furthermore, there are various techniques being explored to load cargo into EVs therefore allowing us to influence their bioactive properties and enhance their effects for specific therapeutic applications. There are two different approaches to influencing EV cargo, exogenous and endogenous loading. Exogenous therapeutic cargo can be loaded into EVs via co-incubation, electroporation or sonication whereas endogenous loading is achieved by genetic modification of the parent cell in order to overexpress a specific nucleotide or protein, which is then incorporated into the secreted EVs[49,194]. Bioengineered MSC-EVs have not yet been explored in preclinical models of neonatal disease however there are good examples of modified MSCs demonstrating heightened therapeutic effects in preclinical models of HIE and PVL such as genetically modified, IFNγ primed and thrombin preconditioned MSCs[30,44,101,103]. There has also been demonstration of good manufacturing practice (GMP) manufactured IFN-γ primed MSCs for potential clinical use with no evidence of toxicity or heightened anti-inflammatory effects[195].

One of the major technical challenges is the absence of standardized criteria of EV production. The ISEV has published a field-consensus standardization of the minimal information for the studies of extracellular vesicles (MISEV) guideline that provides criteria for MSC-EV nomenclature, isolation, purification, and characterization[47]. There continues to be room for improvement with existing isolation methods to yield more homogeneous EV preparations in terms of particle number, potency, and purity, in addition to optimizing the duration and cost of EV production[49]. Another area of concern in production is the need to develop more sensitive tools requiring less of this limited product to quantify and characterize EVs (both phenotypically and functionally). The next MISEV guidelines are currently being established to help provide advice on the pros and cons of different isolation techniques and to develop a panel of markers to differentiate EV subtypes[196]. The optimal source of EVs also needs to be determined given that access to human stem cell sources is limited and there are numerous different types of stem cells that could be utilized to harvest EVs. MSCs have garnered the most attention as they are easily extracted, exert anti-inflammatory effects, have low immunogenicity and self-renewal properties[197]. Most preclinical studies of neonatal disease have demonstrated efficacy utilizing MSCs which can be sourced from a wide range of adult and perinatal tissues. MSCs originating from perinatal tissues (UCB, UC and placenta) have demonstrated increased secretion of chemokines, pro-inflammatory proteins, and growth factors with a higher rate of cellular proliferation compared to MSCs obtained from adult tissues (adipose, BM)[198-200].

Optimal timing and dosing of EVs is also another major query, depending on the clinical scenario single versus multiple doses of MSC-EVs may be required to achieve therapeutic effect. There have been several preclinical studies reporting positive effects with a single dose administered early in the disease process[39,72,73,79,189], while there have been other studies that have shown the need for multiple doses at later time points to ameliorate other

disease processes[72,190]. Other technical challenges in the application of MSC-EVs relates to their administration and storage for clinical purposes. Multiple routes of administration for MSC-EVs have been proposed in pre-clinical models of neonatal diseases with some routes being more high-risk due to their invasive nature such as IC or intravitreal administration. IV administration would be the easiest route from an application standpoint but requires further confirmation of successful delivery to protected organ sites such as the brain and eye. Given that EVs can contain a variety of bioactive molecules in a lipid bilayer, the storage and preservation of the product is essential to maintain its optimal biologic activity[201]. Finally, prior to application in a clinical setting, there needs to be a regulatory framework in place including guidelines regarding GMP, production compliance, quality control criteria, sterilization methods and standard operating procedures for reproducibility[49]. The next MISEV plans to implement further guidelines regarding the safety, efficacy, clinical grade manufacturing, and regulatory issues[196,202]. In addition, the concern regarding scalability of MSC-EV production for use in clinical trials is also being addressed by an increase in biotechnology companies venturing into EV production.

In conclusion, the stem cell secretome (such as MSC-EVs) hold an immense amount of promise as a therapeutic avenue for numerous intractable neonatal pathologies given their immunomodulatory, anti-inflammatory, neuroprotective, angiogenic and regenerative effects (Figure 1). However, further research needs to be done as illustrated above to help develop our understanding of the exact mechanism of action of EVs and refine isolation, characterization, storage, and administration techniques prior to the generation of a robust and clinical-grade EV product for utilization in neonatal patients. Lastly, rigorous clinical trials are still needed to determine both safety and feasibility of EV therapy prior to eventual determination of efficacy and long-term effects.

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Abbreviations:

AF	amniotic fluid
AIS	arterial ischemic stroke
Akt	protein kinase B
APEH	acylaminoacyl-peptide hydrolase
ANXA1	annexin A1
BBB	blood-brain-barrier
Bcl2	B-cell lymphoma 2
BDNF	brain-derived neurotropic factor
BM	bone marrow

BPD	bronchopulmonary dysplasia
Casp3	caspase 3
CBF	cerebral blood flow
CCL2	C-C motif chemokine ligand 2
CCR2	C-C motif chemokine receptor 2
C/EBPα	CCAAT-enhancer-binding protein α
CM	conditioned media
COX-2	cyclooxygenase 2
CP	cerebral palsy
CREB	cAMP response element-binding protein
CX3CR1	C-X3-C motif chemokine receptor 1
EGR2	early growth response 2
ELGAN	extremely low gestational age neonates
EVs	extracellular vesicles
FGR	fetal growth restriction
FOXO3a	forkhead box O3a
FoxP3	forkhead box P3
hAF-MSC	human AF-MSC
hBM-MSC-EVs	human BM MSC-EVs
HI	hypoxic-ischemic
HIE	hypoxic-ischemic encephalopathy
HSCs	hematopoietic stem cells
hUC-MSC-EVs	human UC derived-MSC-EVs
hUCB-MSC	human UCB-MSC
IC	intracerebroventricular
IFNγ	interferon γ
IGF-1	insulin growth factor
κBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

IL	interleukin
IP-10	(interferon-γ induced protein 10
ISCT	International Society for Cellular Therapy
ISEV	International Society for Extracellular Vesicles
IV	intravenous
IVH	intraventricular hemorrhage
LPS	lipopolysaccharide
Ly6C	lymphocyte antigen 6 complex, locus C1
Ly6G	lymphocyte antigen 6 complex, locus G
MAPK	mitogen-activated protein kinase
mBM-MSC-EVs	murine BM derived-MSC-EVs
miR	microRNAs
MISEV	minimal information for the studies of extracellular vesicles
MSC	mesenchymal stem/stromal cell
NEC	necrotizing enterocolitis
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3	NOD-, LRR- and pyrin domain-containing protein 3
NPCs	neural progenitor cells
NSCs	neural stem cells
OGD/R	oxygen/glucose deprivation/reoxygenation
OIR	oxygen-induced retinopathy
PE	preeclampsia
PHH	posthemorrhagic hydrocephalus
PI3K	phosphatidylinositol-3-kinase
PSC	pluripotent stem cells
PTEN	phosphatase and tensin homolog
PVL	periventricular leukomalacia
rBM-MSC-EVs	rodent BM-MSC-EVs

ROP	retinopathy of prematurity
Sema3E	semaphorin 3E
SGK1	serum/glucocorticoid regulated kinase 1
SPC	surfactant protein C
TGFβ	transforming growth factor β
TH	therapeutic hypothermia
Timp3	tissue inhibitor of metalloproteinase 3
TLR-4	Toll-like receptor 4
TNF-α	tumor necrosis factor-a
TrkB	tyrosine kinase B
TSG-6	tumor necrosis factor-stimulated gene-6
UC	umbilical cord
UCB	umbilical cord blood
VEGF	vascular endothelial growth factor
VLBW	very low birth weight
Wnt	Wingless/Integrase 1 family member

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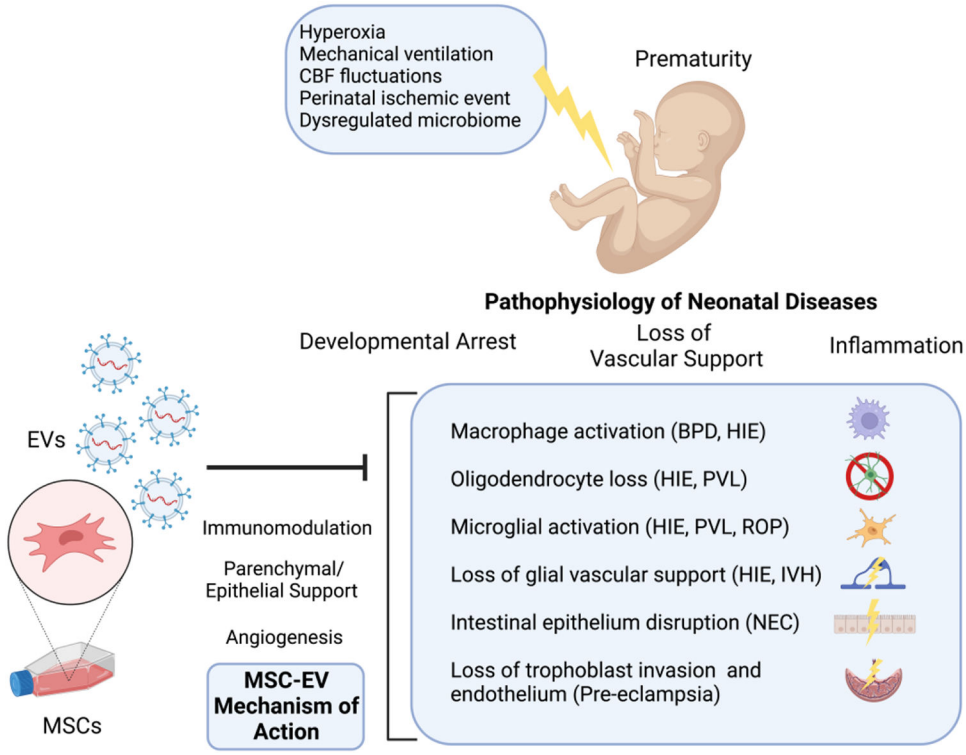

Figure 1. The common pathophysiology shared by neonatal diseases and the mechanism of action of MSC-EV treatment.

Prematurity results in under-development of the lungs, brain, gastrointestinal tract, retina and other vital organs that are susceptible to injury from a variety of sources (such as hyperoxia, mechanical ventilation, cerebral blood flow (CBF) fluctuations, perinatal ischemic events, dysregulated microbiome) that subsequently result in inflammation, developmental arrest and loss of vascular support. MSC-EVs have been shown to exert their therapeutic effects by modulating the immune response and phenotype, maintaining parenchymal and epithelial support and promoting angiogenesis.