Stem cell-based therapy for neonatal lung disease—it’s in the juice

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Abstract

Bronchopulmonary dysplasia (BPD), the chronic lung disease of prematurity, is the most common complication in extreme premature infants (born before 28 weeks gestation). Despite advances in perinatal care, modern clinical management remains devoid of therapies specifically promoting lung repair and lung growth. Recent progress in stem cell biology has uncovered the promise of stem/progenitor cells to repair damaged organs. Contrary to the original theory that stem cells engraft and repopulate the damaged organ, evidence suggests that stem cells act via a paracrine mechanism. This review highlights the pre-clinical evidence for the therapeutic potential of cell-based therapies in animal models of neonatal chronic lung injury and the multiple therapeutic avenues offered by soluble stem cell-derived factors.

INTRODUCTION

The incidence of premature delivery in North America is at 12.5% and rising (1). Prematurity is the leading cause of perinatal mortality and morbidity, placing these neonates at high-risk for long-term medical impairments such as bronchopulmonary dysplasia (BPD). First documented in 1967 by Northway et al., BPD was described as a chronic lung disease following mechanical ventilation and oxygen therapy for acute respiratory failure at birth (2). Since then, antenatal steroids and postnatal surfactant have aided in overcoming the biochemical immaturity of the lung. These advances in perinatal care together with more incremental improvements enabled neonotologists to push back the limits of viability from then 34 weeks gestation to today around 24 weeks gestation. Injury to more immature lungs changed the pathology of BPD (3, 4). Today, BPD is characterized by impaired alveolar development and dysmorphic pulmonary microvascular growth, along with a lesser degree of inflammation and fibrosis compared to the original BPD (5). Injury at these earlier stages...
may be more challenging to prevent and increases the risk of long-term consequences including pulmonary hypertension and early onset emphysema (6), which add to the burden of health care (7, 8). Thus, therapies that promote both lung repair and lung growth are desirable.

Recent insights into stem cell biology promise the regeneration of damaged organs. Stem cells are capable of self-renewal and differentiation into specialized cell types and thus have the potential to promote organogenesis, tissue regeneration, maintenance and repair (9). Mesenchymal stromal cells (MSC) attracted particular interest because of their ease of isolation, characterization, apparent multipotency and pleiotropic effects. Adult bone marrow-derived MSCs (BMSCs) apparently differentiate into cells of various non-hematopoietic tissues. BMSC studies in various disease models, including cardiovascular and neurodegenerative disorders, demonstrated their efficacy in attenuating organ injury (10–13). The demonstration that a bone marrow derived stem cell could differentiate into alveolar epithelial cells ignited stem cell research in the lung (14). Accordingly, pre-clinical studies suggested that bone marrow derived stem/progenitor cells were capable of migrating to the injured lung to promote repair (15) and administration of exogenous BMSCs prevented lung injury in various adult lung disease models (reviewed in Weiss et al. (9)). These studies offered substantial promise to mitigate the impaired alveolar growth in experimental models mimicking BPD. The multipotency and self-renewal of stem cells make cell-based therapies appealing for providing both lung injury prevention and lung growth.

**Cell-based therapies to prevent experimental chronic neonatal lung injury - Proof of concept**

In 2007, Tian et al. showed that intravenous injection of rat BMSCs could ameliorate neonatal lung injury (16). Shortly after, two simultaneously published papers confirmed the therapeutic potential of BMSCs. Intravenously-delivered BMSCs reduced alveolar loss and lung inflammation, and prevented pulmonary hypertension in hyperoxia-induced mice (17). Likewise, intratracheal delivery of BMSCs increased survival and exercise capacity of hyperoxia-exposed rats while attenuating alveolar and vascular injury and pulmonary hypertension (18). Subsequent studies also showed benefits in weight gain (19) and decreased fibrosis (20).

A clinically relevant source of stem cells, especially for the treatment of neonatal diseases, is offered by umbilical cord blood (UCB) (21). UCB contains stem cells that are easily accessible at birth and also capable of differentiating into various cell types (22–24), including alveolar epithelial cells (25). Intratracheal and intraperitoneal administration of human UCB-derived MSCs also improved alveolar growth through various mechanisms (26) and in a dose-dependent manner: $5 \times 10^3$ cells failed to attenuate both hyperoxia-induced lung injury and inflammation, while $5 \times 10^4$ and $5 \times 10^5$ cells attenuated both hyperoxia-induced injuries and inflammatory responses, but the latter dose was more effective (27). Human cord-derived pericytes and UCB-derived MSCs not only prevented but could also repair lung injury in neonatal rats when administered two weeks after established hyperoxia-induced lung injury (28). Long term assessment at six months showed persistent
improvement in lung architecture and exercise capacity and no adverse effects were observed (28).

While MSCs are affirming their promise in regenerative medicine, other stem and progenitor cells are emerging. Amnion epithelial cells prevent antenatal lipopolysaccharide (29)-and ventilation (30)-induced lung injury in fetal sheep. Multipotent amniotic fluid-derived stem cells are capable of differentiating into lineages of all three embryonic germ layers and promote alveolar epithelial cell wound healing and lung growth (31, 32). Consistent with the importance of angiogenesis during lung growth, injury and repair (33), bone marrow-derived angiogenic cells - a novel population of bone marrow myeloid progenitor cells that express angiogenic markers - demonstrated the capacity to restore impaired alveolar and vascular lung growth in hyperoxia-exposed newborn mice (34).

Overall, these observations (summarized in Table 1) provide evidence for the therapeutic benefit of bone marrow and cord blood-derived MSCs in chronic oxygen-induced lung injury in rodents. A recurrent finding, however, is the paucity of cell engraftment, suggesting that stem cell properties such as self-renewal and differentiation are not required for their therapeutic action (35). This finding has led to the hypothesis that MSCs act through a paracrine effect (36), rather than through cell replacement. This realization has expanded the therapeutic options of cell-based therapies.

**MSCs prevent lung injury via a paracrine mechanism - it's in the juice**

Several lines of evidence suggest that MSCs act via a paracrine mechanism to protect the developing lung from injury. In vitro cell-free BMSC conditioned media prevented hyperoxia-induced alveolar epithelial cell apoptosis, accelerated alveolar epithelial cell wound healing and preserved endothelial cord formation on matrigel during hyperoxia (18).

In vivo, the paracrine effect could also be inferred from the efficacy of intraperitoneal administration of MSCs in preventing oxygen-induced neonatal lung injury (20, 26). Accordingly, Aslam et al. provided direct in vivo evidence showing that a single injection of cell-free BMSC-derived conditioned media had a more pronounced effect on alveolar injury and fibrosis prevention than BMSCs themselves (17). In a follow-up study, a single intravenous dose of BMSC-derived conditioned media normalized lung function, reversed alveolar injury and pulmonary hypertension (37). A single intratracheal injection of BMSC or BMSC-free conditioned media protected from oxygen-induced alveolar and vascular injury with a persistent benefit followed up to 3 months (38). Likewise, cell-free conditioned media derived from human UCB-MSCs and pericytes prevented and reversed arrested alveolar growth and lung function in hyperoxic-exposed rats with persistent benefits at 6 months of age and without adverse effect on lung structure or tumour formation (28). Dose-response studies have not yet been performed.

Although the therapeutic benefit of the conditioned media is undeniable, a potential caveat of this strategy is the lack of cell adaptation to the local injurious environment. In an attempt to overcome this potential limitation, Waszak et al. exposed BMSCs to a “deleterious BPD environment” by priming them ex vivo in hyperoxia for 24 hours (39). Conditioned media
collected from these preconditioned cells and injected into hyperoxic rats exerted a more potent therapeutic effect in vivo on lung architecture compared to non-preconditioned media.

Thus, rather than replacing injured cells and differentiating into lung cells, MSCs may release factors that protect resident lung cells from injury or modulate the function of inflammatory cells. Tropea and colleagues recently provided evidence for such a scenario. Bronchio-alveolar stem cells (BASCs) are putative epithelial lung stem/progenitor cells at the bronchio-alveolar junction, capable of self-renewal and differentiation in culture, and also proliferate in response to alveolar injury (40). Both BMSC and BMSC-derived conditioned media increase the number of BASCs in neonatal mice exposed to hyperoxia (41). This study also offers new therapeutic perspectives, i.e. the protection of resident lung progenitor cells rather than exogenous supplementation of stem cells. In addition, there is increasing evidence that MSCs interact with inflammatory cells to modulate their response to injury. MSCs can direct macrophages from a M1 (pro-inflammatory) to a M2 (healer) phenotype in various disease conditions (42, 43). Overall, these observations suggest that cell-free conditioned media exerts similar therapeutic benefit to the cell itself. The exciting challenge is how to harness the multiple healing properties of stem cells.

**MSCs prevent lung injury via a paracrine mechanism - what’s in the Juice?**

Indeed, the identification of soluble factors in the conditioned media may allow the discovery of novel healing molecules that each by itself or in combination could yield new therapeutic options (44). Besides factors already known to be lung protective including keratinocyte growth factor (45), vascular endothelial growth factor (46) or adiponectin (47), novel molecules secreted by MSCs have already been identified and shown therapeutic benefit in various disease models, such as staniocalcin-1 (48) – a potent anti-oxidant – or tumor necrosis factor-alpha-stimulated gene/protein 6 (TSG-6) (49) – a potent anti-inflammatory protein.

From a clinical perspective, a relevant question for the design of clinical trials is to determine the most efficacious and safest stem cell-based approach: whole cell therapy vs. cell free-derived conditioned media vs. identification of single bioactive molecules vs. identification and determination of the most efficacious combination of molecules. This daunting task could be circumvented by the recent recognition that MSCs release membrane vesicles, exosomes in particular, that act as nano-packages containing a combination of bioactive molecules and microRNA (miRNA) (50). miRNA are small non-coding RNA molecules involved in transcriptional regulation of gene expression. In particular, miRNA could become interesting therapeutic targets in the prevention of BPD (51) by silencing specific genes with deleterious effects during lung injury. Exosomes are 40–100 nm in size and represent a specific subtype of secreted membrane vesicles formed through the fusion of multivesicular endosomes with the plasma membrane. Although known for several decades, membrane vesicles have long been thought of as mere cell debris. Recent evidence, however, suggests that MSC-derived exosomes play important roles in cell communication and mediate the therapeutic benefit of MSCs. For example, MSC-derived exosomes attenuate lung macrophage influx, decrease pro-inflammatory cytokine levels in the bronchoalveolar lavage and prevent pulmonary vascular remodeling and hypoxic-induced pulmonary
hypertension in mice (52). With the exosomes removed, the conditioned media showed no therapeutic effect in this model. Similar therapeutic benefits of MSC-derived exosomes are reported in kidney (53) and cardiac (54) injury. A limitation for the exploitation of exosomes as therapeutic tools remains the process of isolation, characterization, quality control, and large-scale manufacturing. Novel findings continue to uncover the mechanisms by which MSCs protect resident lung cells including the transfer of mitochondria via nanotubes (55). These pleiotropic effects (Figure 1) open exciting avenues in particular for multifactorial diseases such as BPD and provide traction for the discoveries of cell-free products.

Considerations for Clinical Trials

A clinical trial testing the safety and efficacy of MSCs in adult patients with COPD has been completed (56). Although this clinical trial was predominantly for safety, no substantial evidence of efficacy of the MSCs was recorded. More recently, a phase I clinical trial testing the safety of human umbilical cord blood derived MSCs in nine premature infants at risk of developing BPD has been completed (ClinicalTrials.gov: NCT01297205). Although this study upholds the safety of MSC therapy, long-term follow-up is warranted. Further clinical trials are already planned (ClinicalTrials.gov: NCT01207869, NCT01828957).

Pre-clinical studies have generated proof of concept evidence that cell-based therapies can prevent and restore experimental neonatal lung injury in rodent and sheep. Rather than cell replacement, the therapeutic benefit of stem cells is mediated through a paracrine effect. It is likely that the combination of bioactive molecules contained in the conditioned media provide the compounding pleiotropic effects attributed to MSCs. Administration of the entire cocktail containing unidentified products may conjure unforeseen side effects and some components may be more beneficial in repair. Thus, further specification of which molecule(s) have reparative properties and/or the isolation of specific micro/nano carriers such as exosomes may lead to pharmacological therapies for BPD.

The cell most suitable for clinical trials appears to be MSCs, likely because of their ease of isolation, characterization and pleiotropic effects. However, endothelial progenitor cells and other stem/progenitor cells have also proven to be effective in pre-clinical BPD models. These various cells differ in their roles and respective factors, therefore possibly producing a more pronounced effect when administered in concert (57), although this remains to be proven in the lung.

Likewise, the source of cells is an important consideration. Umbilical cord and cord blood are easily accessible at birth and may have more potent repair capabilities than adult BMSCs. Autologous UCB-derived cell therapies may avoid immunological risks and allows the use of minimally manipulated cells. However, given the immunological properties of MSCs, allogeneic cell therapy is feasible and may facilitate the logistics of cell-based therapies.

The timing of the treatment is another factor to be resolved. Recent pre-clinical evidence showed UCB-derived MSCs time-dependently attenuated hyperoxia-induced injury, eliciting significant protection in the early, but not the late phase of inflammation (58). In the clinic, the early identification of infants at the most risk of developing BPD through the use of
estimators and models may allow for the selection of an appropriate patient population. Patients with early and persistent pulmonary dysfunction have a close to 70% risk of developing BPD, as defined by Laughon et al. (59), and may represent an at-risk population of choice for cell-based therapies.

Finally, the safety of each of these cell-based therapies must be investigated thoroughly in well-designed pre-clinical trials including large animal models.

In summary, as the incidence of prematurity and chronic neonatal lung disease rises (60), novel therapies are required. Pre-clinical studies have brought substantial promise in developing an effective clinical therapy that could fulfill the dual role of preventing injury and promoting lung growth. The paracrine effect of cell-based therapies has opened unexpected therapeutic options through the identification of individual molecules or mechanisms including microRNA, mitochondrial transfer and microparticles. The promise may not lie in the stem cell itself, but rather in its vast array of bioactive mediators - ‘it’s in the juice’.

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Figure 1.
Schematic representation of possible repair mechanisms associated with stem cells. Many therapeutic mechanisms for stem cells have recently emerged. These include microparticle carriers such as microvesicles, exosomes or multivesicular bodies which are speculated to be released by stem cells and elicit a therapeutic response. MicroRNA packaged in these vesicles or as a sole effector may also play a therapeutic role. The role of secreted soluble proteins/peptides in neonatal and adult lung injury has been extensively studied. This has lead to the discovery of promising bioactive molecules such as the anti-inflammatory IL-10, Staniocalcin-1, TSG-6 and TNF-alpha antagonists, the combination of which may contribute to the pleiotropic effects promoting repair. Recent evidence also unveiled therapeutic mitochondria transfer via nanotubes. These mechanisms can signal endogenous stem cells to amplify or transduce similar repair actions.
### Table 1

| Cell Type          | Source/Route/Control cell | Animal Model               | Age of Animals | Outcomes                                                                                                                                                                                                 | Reference |
|--------------------|---------------------------|----------------------------|----------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| MSC                | BM/IV/no                  | 95% hyperoxia (rat)        | P-10           | Reduced levels of TNF-alpha and TGF-beta1, increased radial alveolar count.                                                                                                                                   | (16)      |
| MSC                | BM/IT/PASMC               | 95% hyperoxia (rat)        | P-14           | *in vitro:* preferential MSC migration toward O2-damaged lungs. MSC-CM prevented O2-induced AEC2 apoptosis, accelerated AEC2 wound healing and enhanced endothelial cord formation.  
*in vivo:* attenuated alveolar and vascular injury, and PH.                                                                                               | (18)      |
| MSC                | BM/IV/no                  | 95% hyperoxia (rat)        | P-3, P-7, P-14 | Improved weight gain, prevented alveolar growth arrest and suppressed lung inflammation.                                                                                                                                 | (19)      |
| MSC                | BM/IP/lung fibroblasts    | 95% hyperoxia (rat)        | P-14           | Preserved alveolar growth. CM from O2-exposed, preconditioned BMSCs exerted more potent therapeutic potential and prevented PH.                                                                                   | (39)      |
| MSC                | BM/IP/no                  | 60% hyperoxia (mouse)     | P-45           | *in vitro:* co-culturing of injured lung tissue increased migration potential of BMSC and SP-C expression.  
*in vivo:* BMSC home to injured lung, express SP-C, improve pulmonary architecture, attenuate pulmonary fibrosis and increase survival rate.   | (20)      |
| MSC                | BM/IP/lung fibroblasts    | 75% hyperoxia (mouse)     | P-14           | Reversed parenchymal fibrosis and peripheral PA devascularisation, partially reversed alveolar injury, normalized lung function, reversed PH and RVH and attenuated peripheral PA muscularization.                          | (37)      |
| MSC                | BM/IP/no                  | 90% hyperoxia (rat)        | P-16, P-33, P-100 | Improved alveolarization and lung vascular growth with MSC and MSC-CM up to 3 months post-treatment. Decreased inflammation and up-regulation of angiogenic factors.                                         | (41)      |
| MSC                | UCB/IT & IP/no            | 95% hyperoxia (rat)        | P-14           | IT & IP: attenuated the increase in TUNEL-positive cells, myeloperoxidase activity, and IL-6 mRNA level.  
IT: improved alveolarization and decreased lung collagen, TNF-alpha and TGF-beta mRNA, alpha-SMA protein.                                                | (26)      |
| MSC                | UCB/IT/no                 | 95% hyperoxia (rat)        | P-14           | Improved alveolarization, decreased lung collagen, and attenuated lung inflammation (decreased myeloperoxidase activity, TNF-alpha, IL-1beta, IL-6, TGF-beta mRNA, up-regulation of cytosolic and membrane p4phox in a dose dependent manner. | (27)      |
| MSC & Pericytes    | Umbilical cord & UCB/IT/  | 95% hyperoxia (rat)        | P-22, P-35, 6 months | Improved alveolarization and lung vascular growth with whole cell and cell-free CM. Prevention and rescue. Efficacy and safety up to 6 months post-treatment.                                                         | (28)      |
| Myeloid progenitor | BM/IV/embryonic EPC, mouse embryonic fibroblasts | 80% hyperoxia (mice)     | P-21           | Restored lung structure.                                                                                                                                                                                   | (34)      |
| Cell Type | Source/Route/Control cell | Animal Model   | Age of Animals | Outcomes                                                                                      | Reference |
|-----------|---------------------------|----------------|----------------|-----------------------------------------------------------------------------------------------|-----------|
| Epithelial| Amnion/IV/no              | LPS (sheep)    |                | Improved lung function and structure (lung volume, tissue-to- airspace ratio, and septal crest density), reduced inflammatory cytokines (TNF-alpha, IL-1beta, IL-6). | (29)      |
| Epithelial| Amnion/IV/no              | Ventilation (sheep) |                | Attenuated lung fibrosis and normalized secondary septal crests. Differentiated into AEC1 and AEC2 in the injured lung. | (30)      |

**Abbreviations:** AEC1/2: alveolar epithelial type I/II cell, BASC: bronchioalveolar stem cells, BM: bone marrow, CM: conditioned media, EPC: endothelial progenitor cell, IV: intravenous, IT: intratracheal, IP: intraperitoneal, LPS: lipopolysaccharide, P-\(n\): postnatal day-\(n\), PA: pulmonary artery, PASMC: PA smooth muscle cells, PH: pulmonary hypertension, RVH: right ventricular hypertrophy, SMA: smooth muscle actin, SP-C: surfactant protein C, TNF: tumor necrosis factor, TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling, UCB: umbilical cord blood.