Bronchopulmonary dysplasia (BPD) is a complex chronic lung disease (CLD) with multifactorial etiology, characterized by the arrest of alveolar and vascular growth associated with inflammation and parenchymal fibrosis.\(^1\)\(^-\)\(^4\) Historically, oxygen toxicity and ventilator-induced injury have been the prerequisites for BPD in premature infants born at less than 28-32 weeks gestation with respiratory distress syndrome, but BPD may also occur in immature infants with few signs of initial lung injury. Approximately one in four infants with moderate-to-severe BPD develops pulmonary hypertension (PH)\(^5\) that can be triggered by inflammation and endothelial dysfunction, and perpetuated through alveolar hypoxia. Importantly, the occurrence of PH in BPD is not only an epiphenomenon or minor secondary event, but appears to greatly increase mortality (estimated death rate is 47% two years after diagnosis of PH).\(^6\) Moreover, with an increasing percentage of extremely immature newborns surviving in the post-surfactant era, BPD is one of the most common primary diagnoses in neonatal follow-up and pediatric...
The saccular stage of murine lung development is completed after two weeks of postnatal alveolarization. Hence, the developmental stage of the mouse lung at birth resembles that in the human preterm neonate between 24 and 28 weeks gestation, making the newborn mouse an excellent model to study human developmental lung injury. Hyperoxia-induced lung damage in neonatal mice is characterized by rarefication and simplification of alveoli and thickened alveolar septa, inflammation and parenchymal fibrosis, a pattern which is similar to human BPD.\[8\]

Mesenchymal stem cells, also referred to as multipotent stromal cells (MSCs), have attracted significant attention as potential cell-based therapy for BPD and other severe lung diseases because these multipotent cells exhibit beneficial effects in related animal models through anti-inflammatory, immunomodulatory, pro-survival (endothelial, epithelial), and anti-fibrotic mechanisms.\[9,10\] We and others have previously shown that administration of bone marrow-derived MSCs prevent BPD/CLD of prematurity,\[11,12\] acute lung injury,\[13,14\] and pulmonary fibrosis\[9\] in experimental models despite low engraftment rates. Our previous study revealed that conditioned media (CM) from MSCs was most effective in preserving alveolar architecture in the hyperoxia-BPD mouse model and suggested that bone marrow-derived MSCs have important cytoprotective effects predominantly via paracrine mechanisms.\[11\]

Although innovative approaches for the prevention of mechanical ventilation, oxidative stress, and ultimately BPD have been developed recently,\[15-17\] larger studies have failed so far to demonstrate an efficient preventative or therapeutic strategy for BPD in preterm newborn infants.\[18\] From a clinical perspective, reversal rather than postnatal prevention of oxygen-induced lung injury such as BPD is most relevant, given that (1) BPD probably has prenatal origins (preclampsia,\[19,20\] oligohydramnios,\[5\] chorioamnionitis\[21,22\]), and (2) hyperoxic lung injury occurs in the first few minutes and hours of life when endotracheal intubation, mechanical ventilation, and oxidative stress cannot be avoided due to respiratory failure.\[15,23\]

While the human umbilical cord or placenta may serve as a source for future clinical MSC applications,\[24\] we herein used CM from cultured mouse bone marrow-derived MSCs as a feasible intravenous treatment in the well-established murine hyperoxia-BPD model. We found that several weeks after injury, MSC-CM-treatment (1) reversed the parenchymal fibrosis and peripheral pulmonary arterial devascularization (PA pruning), (2) partially reversed the alveolar injury, (3) normalized the airway hyperresponsiveness, (4) fully reversed the moderate PH and right ventricular hypertrophy (RVH), and (5) attenuated peripheral PA muscularization that was associated with BPD. Thus, hyperoxia-induced BPD and associated PH can be reversed with MSC-CM-treatment, thereby pointing toward a new therapeutic approach for chronic lung diseases with alveolar and vascular injury. Importantly, we show that a single intravenous dose of MSC-CM after the acute phase of lung injury in the neonatal period inhibits longterm impairment of lung function, an increasingly recognized serious complication of BPD.

**MATERIALS AND METHODS**

**Experimental design**

Mouse pups (FVB) were exposed to 75% oxygen from postnatal day 1 (P1) to P14 (Fig. 1, upper panel), or remained in room air from birth (normoxic controls). On P14, when chronic hyperoxic lung injury is evident in this BPD model,\[8,11\] the mice were placed in room air, and intravenously injected with concentrated MSC-CM containing 10 µg protein, or the equivalent amount of mouse lung fibroblast-CM (MLF-CM) as control. Two weeks after the end of hyperoxia, i.e., after two weeks recovery in room air at postnatal age four weeks, lung tissue was harvested for histology and immunohistochemistry as described below. Four weeks after the end of hyperoxia, i.e., at postnatal age six weeks and body weight of 18-22 grams, all mice (including normoxic controls) underwent echocardiography and pulmonary function tests. All animal experiments were approved by the Children’s Hospital Boston Animal Care and Use Committee.

**Hyperoxia chamber**

Neonatal pups were pooled and exposed to 75% oxygen in a plexiglass chamber or to room air beginning at birth and continuing for 14 days. Ventilation was adjusted by an Oxycycler controller (Biospherix, Lacona, N.Y.) to remove CO₂ so that it did not exceed 5,000 ppm (0.5%). Ammonia was removed by ventilation and activated charcoal filtration through an air purifier. Dams were rotated from hyperoxia to room air every 48 hours to prevent excessive oxygen toxicity to the adult mice.

**Cell isolation, culture, and differentiation**

Bone marrow-derived MSCs were isolated from the femurs and tibiae of 5- to 7-week-old FVB mice as previously described.\[11,25-27\] Briefly, the bone marrow cells were
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Figure 1: MSC-CM-treatment partially reverses neonatal hyperoxia-induced alveolar injury, septal thickening, collagen accumulation, myofibroblast infiltration, and inflammation. Upper panel: Experimental Design. Newborn mice were either left in room air, or exposed to hyperoxia (FiO₂=0.75) for 2 weeks (P1-14), and then intravenously injected once with CM from either bone marrow-derived MSCs or primary MLFs, and placed in room air for 2 additional weeks, followed by harvest, inflation and fixation of lungs. Lung sections were stained for hemotoxylin & eosin (A, C, E; 100×), and Mason Trichrome (B, D, F; 400×). Inserts were taken at 200× (H&E) and 400× (Mason Trichrome) magnification to illustrate interstitial inflammation (C, arrows), inflammation and fibrosis (D). Severe destruction of the alveolar architecture with overall widened airspaces and interstitial infiltration of inflammatory cells (macrophages, leukocytes) and myofibroblasts was seen in the hyperoxia-exposed/MLF-CM-treated mice (C, D) when compared to normoxic controls (A, B). Enhanced collagen deposition was seen in myofibroblasts, alveolar septa, and perivascular spaces in hyperoxia-exposed/MLF-CM-treated mouse lungs (D, arrows and inset). These changes were absent or greatly abrogated in MSC-CM-treated mice that had honeycomb-like alveoli (E, F) similar to normoxic controls, with residual emphysema. Quantification of mean linear intercept (Lm) as a surrogate of average air space diameter (G) and collagen content (H) are shown in the lower panels. Mean±SEM, n=4-7 per group, ***P<0.001, **P<0.01, *P<0.05. Scale bar = 100 µm (A, C, E) and 50 µm (B, D, F).
layered on a Ficoll-Paque (Amersham, Piscataway, N. J.) density gradient, centrifuged, and plated. Plastic adherent cells were maintained in culture with α-MEM changed every two to three days. Following two to three passages, immunodepletion was performed as per published protocols and the International Society for Cellular Therapy (ISCT) guidelines.[28] The cells were negatively selected for CD11b, CD14, CD19, CD31, CD34, and CD45, and CD79α antigens using the appropriate fluorescently tagged antibodies (BD Biosciences, Pharmingen, San Diego, Calif.) in a fluorescence-activated cell sorter (MoFlo, Beckman-Coulter), further propagated, and then positively selected for CD73, CD90, CD105, c-kit, and Sca-1 antigens, as described. The differentiation potential of MSC cultures was assessed following published protocols.[26,27] Primary MLFs were derived according to the standard methods.[29]

**Preparation of mesenchymal stem cell-conditioned media**

Bone marrow-derived MSC confluent cultures were incubated in serum-free α-MEM media for 24 hours and the CM from equal numbers of cells in each culture were concentrated 10-fold using an Amicon Ultra Centrifugal Filter Device (Millipore Corporation, Billerica, Mass.) with a molecular weight cut-off of 10 kDa. MLF-CM in the same concentration and volume served as control. Cells were used for derivation of conditioned media between passages 8-10.

**Intravenous injection of MSC- and MLF-conditioned media**

A volume of 50 µl of MSC- or MLF-derived CM concentrate, equivalent to a total of 10 µg MSC-CM protein per mouse, was injected via the superficial temporal vein or the jugular vein on P14. Under these conditions, survival of injected pups was greater than 90%.

**Echocardiography**

Anesthesia was induced with isoflurane 3% and continued at 1–2% to a goal heart rate of 350–450 bpm. Transthoracic echocardiography was performed in anesthetized mice (FiO₂ 1.0) using the Vevo 2100 machine (Visual Sonics, Toronto, Calif.) and a 40 MHz linear transducer with simultaneous ECG recording. In the anteriorly angulated left parasternal long axis view, PW Doppler was applied to measure the pulmonary artery acceleration time (PAAT) and the PA ejection time (PAET). A short PAAT or small PAAT/PAET ratio indicates high peak systolic PA pressure, as previously described and validated.[16,31] In the right parasternal long axis view, M-mode was applied to determine RV anterior (free) wall thickness (RVWT) using a virtual guideline across the RV free wall and RV cavity that cuts through the mitral valve annulus.

**Pulmonary function tests**

To evaluate lung function, mice were anesthetized with 80 mg/kg pentobarbital sodium i.p. Following tracheostomy, mice were mechanically ventilated at a rate of 150 breaths/minute, a tidal volume of 10 ml/kg, and a positive end-expiratory pressure (PEEP) of 3 cmH₂O with a computer-controlled small animal ventilator (Scireq, Montreal, Canada). To evaluate bronchial hyperresponsiveness, normal saline alone and escalating doses of inhaled methacholine (1.6, 5, 16, and 50 mg/ml) were aerosolized by using an ultrasonic nebulizer (Scireq, Montreal, Canada). Average airway resistance was calculated at baseline and maximal values were recorded after each dose of methacholine. In addition, dynamic lung compliance (Cdyn) was determined at baseline and minimum values after each methacholine dose were recorded.

**Lung tissue perfusion and histology**

Following terminal anesthesia with 240 mg/kg Avertin i.p., lungs were perfused with PBS through the right ventricle at a constant pressure of 25 cmH₂O. Lungs were inflated to a fixed pressure of 15–20 cmH₂O with 4% paraformaldehyde and postfixed overnight, subsequently processed, and paraffin embedded for sectioning.

**Immunohistochemical staining**

Lung tissue sections were deparaffinized in xylene and rehydrated. Immunohistochemical analysis was performed by incubating with the indicated primary antibody at a dilution of 1:200 (von Willebrand factor, vWF; rabbit polyclonal anti human; Dako), 1:125 (alpha smooth muscle actin, α-SMA; mouse monoclonal; Sigma) overnight at 4°C after 20 minutes of blocking at room temperature to reduce nonspecific binding. Endogenous peroxidase activity was inhibited with 0.3% H₂O₂ in methanol (Sigma). Secondary antibodies and peroxidase staining was performed according to manufacturer’s instructions (Vector laboratories, Burlingame, Calif.). Slides were counterstained with methyl green.

**Lung parenchymal and pulmonary vascular morphometry and quantification**

Lung sections were stained by Hematoxylin & Eosin (H&E) and Mason Trichrome (collagen). Ten randomly selected areas from 5 µm H&E stained lung sections were captured at 100× (H&E) and 400× (Mason Trichrome) magnification using a Nikon Eclipse 80i microscope. Calibrations for the images were done by acquiring standard micrometer images using the same magnification. Large airways and vessels were avoided for the lung morphometry. The air space chord length (Lm) was calculated using Metamorph image analysis software (Molecular Devices, Sunnyvale, Calif.). The images were superimposed on a grid with parallel lines
RESULTS

**MSC-CM-treatment reverses alveolar injury, septal thickening, and myofibroblast infiltration associated with hyperoxia-induced lung Injury**

Newborn mice exposed to two weeks of hyperoxia followed by a single dose of non-MSC control conditioned media (MLF-CM) showed severe destruction of the alveolar architecture with overall widened airspaces, alveolar simplification, airway remodeling, and interstitial infiltration of inflammatory cells (macrophages, neutrophils) and myofibroblasts, when compared to normoxic mice at four weeks of age (Fig. 1A–F). In the MLF-CM-treated animals, the many myofibroblasts within the alveolar walls had a high collagen content and frequently dendritic extensions elongating along the destructed alveolar remnants thereby creating ellipsoid to round structures (Fig. 1C and D). Collagen deposition, that was practically absent in normoxic mice, was also seen in alveolar septal and perivascular spaces of hyperoxia-exposed/MLF-treated mouse lungs (Fig. 1D). These hallmarks of dysfunctional pulmonary regeneration and fibrosis after hyperoxia in MLF-CM-treated animals were absent or greatly ameliorated in MSC-CM-treated mouse lungs that had honeycomb-like alveoli similar to normoxic controls (Fig. 1E and F). To quantify the effect of MSC-CM on hyperoxia-induced lung damage, septal collagen content, alveolar septal thickening, and Lm, as an approximation of alveolar air space diameter, were determined. MSC-CM-treatment after two weeks of hyperoxia decreased the deposition of alveolar septal collagen by 50% when compared to the hyperoxia-exposed/MLF-CM-treated animals (3.75±1.92 vs. 7.51±0.89% collagen staining of total septal area; \(P<0.01\); Fig. 1H). Alveolar septal thickness, a combined variable of interstitial edema, inflammation, and parenchymal fibrosis, was quantified by measuring the fiber breadth (area/length). Septal thickening was evident in hyperoxia-exposed/MLF-CM-treated neonatal mice versus normoxic controls (3.92±0.07 vs. 3.36±0.20 \(\mu\)m; \(P<0.05\)), and ameliorated in animals that were treated with a single dose of MSC-CM (3.63±0.05 \(\mu\)m; Fig. 1E and F).

The mean alveolar chord length was approximated by Lm measurements and was found to be increased by 97% in hyperoxia-exposed/MLF-CM-treated mice when compared to normoxic controls (Lm 62.4±2.5 vs. 31.7±1.0 \(\mu\)m; \(P<0.01\)). Hyperoxia-exposed/MLF-CM-treated mice had significantly smaller airspaces (Lm 55.3±1.7 \(\mu\)m; \(P<0.01\)) than MLF-CM-treated animals, but moderate residual emphysema four weeks postnatally when compared with normoxic controls (\(P<0.01\); Fig. 1G). Thus, a single intravenous dose of MSC-CM improved the alveolar simplification, inflammation, and fibrosis associated with hyperoxia-induced BPD resulting in...
moderate residual alveolar emphysema but otherwise near-normal lung structure.

**MSC-CM improves lung function after hyperoxia-induced lung injury**

To determine the functional impact of the histological findings, we performed pulmonary function testing in 6-week-old mice 4 weeks after the end of hyperoxia, and in age-matched normoxic mice. All animals were ventilated at a PEEP of 3 cm H₂O. Airway resistance was measured at baseline and after serial methacholine doses in order to quantify bronchial hyperreactivity. At baseline, the airway resistance was not different between the three groups (Fig. 2A). Intriguingly, MSC-CM-treatment fully reversed the abnormal increase in airway resistance response from low to high intratracheal methacholine doses (5, 16, 50 mg/ml) seen in the MLF-CM group to levels not different from normoxic controls (Fig. 2A). At a dose of 50 mg/ml intratracheal methacholine, dynamic lung compliance was greatly decreased in the hyperoxia-exposed/MLF-CM-treated mice when compared to normoxic controls, but was normal in the hyperoxia-exposed/MSC-CM-treated animals (Fig. 2B). Thus, MLF-CM-injected mice exposed to high oxygen concentrations had severe airway hyperresponsiveness to inhaled methacholine, consistent with our histological observations of airway remodeling and myofibroblast infiltration (Fig. 1C and D). However, MSC-CM-treated mice had normal dynamic lung compliance, and normal airway resistance (bronchial reactivity) responses to methacholine (Fig. 2), consistent with the histological findings (Fig. 1E and F vs. 1C and D).

**MSC-CM-treatment reverse pulmonary hypertension and RV hypertrophy in hyperoxia-induced lung injury**

To assess the effects of hyperoxia and MSC-CM on both RV mass and PA pressure, M-mode and PW-Doppler echocardiography were applied. Compared with normoxia (Fig. 3A), the PAAT and PAAT/PAET ratios (i.e., surrogates of mean PA pressure) were found to be decreased in hyperoxia-exposed/MLF-CM-treated animals (Fig. 3C), but improved to normoxic values in hyperoxia-exposed/MSC-CM-treated mice (Fig. 3E) (PAAT: 10.87±0.45 vs. 14.92±0.36 vs. 15.22±0.34 ms in normoxic controls, P<0.001; PAAT/PAET ratio: 0.174±0.007 vs. 0.235±0.005 vs. 0.231±0.008 ms in normoxic mice, P<0.001). In accordance with the reversal of PH after MSC-CM-treatment, we found thickening of the RV free wall (i.e., RVH) in the hyperoxia-exposed/MLF-CM-treated mice (Fig. 3D) that was normalized in the MSC-CM-treated animals (Fig. 3F) (RVWT: 0.354±0.008 vs. 0.259±0.016 vs. 0.235±0.013 mm in normoxic mice, P<0.001). Thus, a single dose of MSC-CM reversed the moderate pulmonary hypertension and RV hypertrophy that was associated with hyperoxia-induced BPD (Fig. 3G and H).

**MSC-CM attenuate hyperoxia-induced peripheral pulmonary arterial remodeling**

To explore the potential impact of MSC-CM on hyperoxia-induced peripheral pulmonary vascular muscularization, random lung sections were stained for the smooth muscle marker, α-SMA. The hyperoxia-induced enhancement of peripheral PA muscularization was already evident in the Mason Trichrome stained lung sections in the MLF-CM group (Fig. 1D) and confirmed by quantification of the SMA staining (Fig. 4). Accordingly, the medial thickness index was 46.3±1.8 versus 17.1±0.8 in normoxic controls (P<0.001; Fig. 4B and D). A single dose of MSC-CM at the end of chronic hyperoxia ameliorated the abnormal peripheral PA muscularization seen in the
MLF-CM-treated mice exposed to high inspiratory oxygen concentrations (medial thickness index 40.8±1.7, \(P<0.05\); Fig. 4C and D).

**MSC-CM rescue hyperoxia-induced loss of peripheral pulmonary blood vessels**

In order to determine whether the loss of pulmonary vessels with chronic high oxygen exposure can be improved or reversed by a single dose of MSC-CM, quantification of pulmonary blood vessels of less than 50 and 50–100 µm diameter was performed in vWF-stained lung sections. Hyperoxia led to significant loss of small vessels of less than 50 µm diameter in the MLF-CM group (Fig. 5B and D), whereas MSC-CM injection restored the small (peripheral) vessels two weeks in recovery from chronic exposure to 75% oxygen (\(P<0.01\); Fig. 5C and 5D). There was also a clear trend toward a protective effect of MSC-CM-treatment on the number of moderate-sized vessels (50–100 µm) (MSC-CM vs. MLF-CM; \(P=0.0534\); Fig. 5D). The counts of larger vessel were low and there was no significant difference in the quantity of vessels >100 µm diameter between the groups (data not shown).

**MSC-CM reverse pulmonary artery pruning in hyperoxia-induced lung injury**

To confirm the histological findings on the rescue of pulmonary vessel loss with MSC-CM-treatment, we performed PA barium injections and subsequent CT-3D reconstruction of the PA vascularization. In accordance with the histological evidence of peripheral vessel loss after chronic hyperoxia we found severe rarefication of peripheral pulmonary arteries in lung CT scans of PA barium injected mouse lungs in the hyperoxia-exposed/MLF-CM-treated (control) group at four weeks recovery in room air (PA pruning; Fig. 6B). Hyperoxia-induced PA pruning was fully reversed by a single intravenous dose of MSC-CM given at the end of hyperoxia at P14, indicating a remarkable angiogenic/vasculogenic effect of MSC-CM (Fig. 6C).

**DISCUSSION**

Chronic lung diseases such as BPD and pulmonary fibrosis will be the second leading cause of death.
Pulmonary inflammation and damage. Preterm infants are predominantly affected by the disease because of their underdeveloped airway supporting structures, surfactant deficiency, decreased lung compliance, and decreased antioxidant capacity. In the post-surfactant era, the pathobiology and clinical course of BPD has changed, and the disease is now characterized mainly by (1) impaired alveolarization with fewer, larger, and simplified alveoli, and (2) dysmorphic vasculogenesis, resulting in fewer (small) pulmonary arteries and frequently pulmonary hypertensive vascular disease that impacts survival. Additional features of the “new BPD” include inflammation, bronchial smooth muscle thickening, and interstitial edema. Pulmonary hypertensive vascular disease in BPD is characterized by pulmonary arteriolar muscularization, vessel loss, and RV hypertrophy, among other findings. Nearly all of these histological features— including excess myofibroblast proliferation and septal collagen deposition— were evident in the murine hyperoxia-BPD model used in this study.

To date, all available interventions for the prevention and/or treatment of BPD have not been effective in randomized controlled trials or have unacceptable adverse effects (e.g., postnatal glucocorticoids). Prevention of premature birth and elimination of prenatal risk factors for BPD, such as preeclampsia and chorioamnionitis, is desirable but difficult to achieve. Therefore, finding an effective treatment approach for BPD and associated pulmonary vascular disease is of tremendous clinical importance.

There is emerging evidence from animal and clinical pilot studies that stem cell and progenitor cell-based therapies modulate disease markers and may be efficient in tissue/organ regeneration. Bone marrow-derived MSCs have been shown to be efficient in the repair of heart and lung diseases such as myocardial infarction, pulmonary fibrosis, and LPS-induced lung injury. Bone marrow-derived MSCs and MSC-CM from mice and rats, or human cord blood-derived MSCs, when given intravenously or intratracheally in a preventive fashion, have been shown to improve lung architecture in rodent models of hyperoxia-induced BPD.

Previously, we demonstrated that intravenous injection of bone marrow-derived MSCs in newborn mice conferred significant vascular and immunological protection from hyperoxia-induced injury but had limited effect in preserving alveolar architecture. Concentrated MSC-CM, however, prevented both vascular and alveolar hypoxic injury resulting in normal alveolar number and thin septa, comparable to controls in room air. The results of our previous preventive approach suggested that bone marrow-derived MSCs have important cytoprotective effects in the hyperoxia mouse model of developmental lung injury worldwide by 2020. BPD is a complex disease of premature infants with multiple pre and postnatal risk factors, including infection, preeclampsia, postnatal oxygen toxicity, and barotrauma, ultimately leading to pulmonary inflammation and damage.
In the current study, we showed that a single intravenous dose of MSC-CM reversed – to a significant degree – hyperoxia-induced BPD and pulmonary vascular disease versus MLF-CM control: MSC-CM-treatment (1) reversed the hyperoxia-induced parenchymal fibrosis and peripheral PA devascularization (PA pruning), (2) partially reversed alveolar injury, (3) normalized lung function (airway hyperresponsiveness, dynamic lung compliance), (4) fully reversed the moderate PH and RVH, and (5) attenuated peripheral PA muscularization associated with hyperoxia-induced BPD.

The paracrine effects of MSCs include the release of immune and growth modulators identified in the CM by mass spectroscopy analysis.[11] These factors in MSC-CM promote signaling pathways of lung repair and include inhibitors of inflammation that are linked to the development of PH and pulmonary fibrosis. An attractive speculation is that the beneficial effect of MSC-CM may be, at least in part, due to activation of endogenous bronchioalveolar stem cells (BASCs), an adult lung stem cell population capable of self-renewal and differentiation in culture, and proliferation in response to bronchiolar and alveolar lung injury in vivo. Very recently, Tropea et al.[38] have demonstrated that intravenous treatment of neonatal hyperoxia-exposed mice with MSCs or MSC-CM led to a significant increase in BASCs compared to untreated controls. Treatment of BASCs with MSC-CM in culture resulted in an increase in growth efficiency, suggesting a paracrine effect of MSCs on BASCs. Lineage tracing in bleomycin-treated adult mice showed that CCSP-expressing cells, including BASCs, are capable of contributing to alveolar repair after lung injury. Thus, MSCs and MSC-derived factors probably stimulate BASCs to contribute to the restoration of distal lung cell epithelia in BPD.[38]

Several studies have highlighted the existence of different MSC phenotypes in bone marrow, blood, and airways/lung. MSC phenotypes and function likely depend on maturity (gestational age) and environmental factors such as tissue oxygenation, and are distinctly regulated by the two major pathways of MSC differentiation, i.e., transforming growth factor beta (TGFβ) superfamily and canonical Wnt pathways.[35,39] Along these lines, in vitro treatment of airway MSCs of ventilated preterm infants with recombinant TGFβ1 induced myofibroblast differentiation, whereas adult human bone marrow-derived MSCs that were not exposed to high oxygen concentrations failed to undergo such differentiation upon TGFβ1 stimulation.[40] Popova et al. concluded that neonatal lung MSCs demonstrate an expression pattern characteristic of myofibroblastic progenitor cells (mRNAs encoding contractile and extracellular matrix proteins, and expression of α-SMA, MHC, and SM22 protein). Conditioned media from cultured tracheal aspirate MSCs of preterm infants exposed to hyperoxic stress contain

![Figure 5: MSC-CM rescue hyperoxia-induced loss of peripheral pulmonary blood vessels. Lung sections were stained for the endothelial cell marker vWF. vWF-positive vessels between 25 and 200 µm outer diameter were counted at 200× magnification in 10–15 random views as described under Methods. Compared to normoxic controls (A), hyperoxia exposure led to significant loss of small vessels < 50 µm diameter in the MLF-CM group (B), whereas MSC-CM injection (C) restored small vessels after 2 weeks in recovery from chronic exposure to 75% oxygen (see Fig. 1 for experimental design). There was also a clear trend toward MSC-CM-treatment effect on the number of larger vessels (50–100 µm) in mice exposed to hyperoxia (MSC-CM vs. MLF-CM; P=0.0534). Quantification of pulmonary blood vessels of less than 50 and 50–100 µm diameter in vWF-stained lung sections (D) was performed as described under Methods. There was no significant difference in numbers of larger vessels (100–200 µm) between the groups. See Figure 1 for experimental design. Mean±SEM, n=4–7 per group, ** P<0.01, * P<0.05. Scale bar = 50 µm.](image-url)
It was proposed that the tracheal organs, and arise from both small and large arteries, shown to reside in the perivascular compartment of many organs.

Besides the bone marrow, multipotent MSCs have been reported in whole lung tissue of neonatal mice with hyperoxia-induced BPD and is associated with fibrosis. Differentiation, release from bone marrow, tissue migration, and the predominant actions of EPCs in peripheral tissues/organs might be regulated by MSCs through paracrine mechanisms, as recently shown for BASCs (see above). In particular, the macrophage-colony stimulating factor (M-CSF) and granulocyte-colony stimulating factor (G-CSF) accelerate neovascularization in vivo and induce differentiation of bone marrow cells into endothelial progenitor cells in vitro.

Recently experimental and clinical data suggest that hyperoxia induces BPD in premature newborns by impairing the number and in vivo functionality of circulating (blood), lung, and bone marrow endothelial progenitor cells (EPCs). The quantity of circulating EPCs is low at extremely low gestational ages and decreases during gestation. Importantly, extremely preterm infants who display lower EPC numbers in blood at birth have an increased risk of developing BPD.

Recent experimental and clinical data suggest that hyperoxia induces BPD in premature newborns by impairing the number and in vivo functionality of circulating (blood), lung, and bone marrow endothelial progenitor cells (EPCs). The quantity of circulating EPCs is low at extremely low gestational ages and decreases during gestation. Importantly, extremely preterm infants who display lower EPC numbers in blood at birth have an increased risk of developing BPD.

Currently, it is unclear whether increased TGFβ1 signaling and subsequent MSC to myofibroblast transdifferentiation occurs as a direct response to hyperoxia in the human preterm lung.

Importantly, increased expression of TGFβ1 and Wnt has been reported in whole lung tissue of neonatal mice with hyperoxia-induced BPD and is associated with fibrosis in BPD and several other CLD models. However, alveolar myofibroblasts are required for the formation of secondary septa during normal lung development, a process that is arrested in BPD, indicating that the timing and adequate regulation of MSC differentiation is crucial for normal lung development.

Besides the bone marrow, multipotent MSCs have been shown to reside in the perivascular compartment of many organs and arise from both small and large arteries as well as capillaries. It was proposed that the tracheal aspirate MSCs found in one study in preterm infants who later on develop BPD are derived from the perivascular tissue of pulmonary and bronchial arteries.

We speculate that hyperoxia-induced TGFβ1 expression and secretion from airway/perivascular MSCs and other lung cells switches the phenotype of pulmonary MSCs and other proliferative cells toward pathological myofibroblast transdifferentiation resulting in dysfunctional repair (alveolar collagen accumulation/fibrosis and simplification, airway and pulmonary vascular remodeling, inflammation), consistent with our histological findings. The report by Popova et al. suggests that adult bone marrow-derived MSCs may be rather resistant to profibrotic stimuli such as hyperoxia and TGFβ1. Our study shows that bone marrow-derived MSCs secrete factors with antifibrotic, antimitogenic, and anti-inflammatory effects that may regulate signaling downstream of the TGFβ receptor and inhibit myofibrogenic transdifferentiation of neonatal lung MSCs and/or alveolar epithelial type II cells. This is supported by our previous analysis of the MSC secretome.

Possible risks of stem cell therapy include the potential for tumor formation and fibrosis. Indeed, fibrocytes, a pool of circulating mesenchymal precursors which can differentiate into myofibroblasts, were reported to be recruited to the lung and contribute to fibrosis as well as pulmonary adventitial remodeling in experimental PH. The human bone marrow, umbilical cord (tissue, blood), or placenta might serve as a source for MSC/MSC-CM and probably will be used clinically as rescue therapy for BPD and other CLD in the near future, when safety of such interventions can be confirmed and the mechanisms of the important paracrine effects of MSCs are better understood.
MSC-CM, the immunological responses to autologous or allogeneic MSC transplantation, certain compounds of MSC-CM, and the best source and route of administration need to be explored more comprehensively.

This study shows that a single intravenous dose of bone marrow-derived MSC-conditioned media rescues hyperoxia-induced BPD by reversing lung parenchymal fibrosis, pulmonary hypertension, vessel loss (PA pruning), and RV hypertrophy, significantly decreasing alveolar injury, reversing airway hyperresponsiveness, and normalizing dynamic lung function long-term. While the mechanisms underlying the beneficial effects of MSC-conditioned media will need to be explored in subsequent studies, MSC-derived interventions appear to be a promising treatment option for BPD, pulmonary hypertension, and other chronic lung diseases, and should be investigated in future clinical trials.

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