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Bronchopulmonary dysplasia: Past, current and future pathophysiologic concepts and their contribution to understanding lung disease

Edited by
Anne Hilgendorff and Andrew Bush

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Bronchopulmonary dysplasia: Past, current and future pathophysiologic concepts and their contribution to understanding lung disease

Topic editors

Anne Hilgendorff — Ludwig Maximilian University of Munich, Germany

Andrew Bush — Imperial College London, United Kingdom

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EDITORIAL
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Editorial: Bronchopulmonary Dysplasia: Past, Current and Future Pathophysiologic Concepts and Their Contribution to Understanding Lung Disease

Andrew Bush[1,2,3] *and Anne Hilgendorff*[4,5,6*]

[1] *Imperial Centre for Paediatrics and Child Health, London, United Kingdom,* [2] *National Heart and Lung Institute, London, United Kingdom,* [3] *Royal Brompton and Harefield NHS Foundation Trust, London, United Kingdom,* [4] *Center for Comprehensive Developmental Care (CDeC*[LMU]*) at the Interdisciplinary Social Pediatric Center, Department of Pediatrics, Dr. von Hauner Children's Hospital, University Hospital, LMU Munich, Ludwig-Maximilians University, Munich, Germany,* [5] *Institute for Lung Health and Immunology and Comprehensive Pneumology Center, Helmholtz Zentrum München, Munich, Germany,* [6] *German Center for Lung Research (DZL), Giessen, Germany*

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Editorial on the Research Topic

Bronchopulmonary Dysplasia: Past, Current and Future Pathophysiologic Concepts and Their Contribution to Understanding Lung Disease

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Laurent Pierre Nicod,
University of Lausanne, Switzerland

***Correspondence:**
Anne Hilgendorff
anne.hilgendorff@med.uni-muenchen.de

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Pulmonary disease arising from both pre- and immediate postnatal adverse events affecting the developing lung was first described around 50 years ago by Northway and colleagues (1). By today's standards, the disease termed "bronchopulmonary dysplasia" (BPD) was seen in relatively mature babies weighing over 2 kg at birth, who were ventilated with higher pressures and at slower rates than would be acceptable today. Much has happened since then.

Whereas, the initial or so called "old" BPD (Northway) was characterized by a pronounced effect on the airways, albeit with an element of failure of alveolar development, BPD today ("new" BPD) is characterized by lung hypoplasia driven by dysregulated growth factor signaling (2) in the context of extensive matrix remodeling and a pronounced inflammatory response (3, 4). This picture arises because neonatologists worldwide now salvaging babies weighing <500 g at birth.

Despite improved perinatal care, and most likely reflecting the changing picture of structural and functional consequences of lung injury in this patient cohort, the overall rates of chronic lung disease in the preterm infant have not decreased significantly and BPD remains the most common morbidity of prematurity (5–7). Adding another level of complexity, the validity and utility of commonly used BPD definitions have been questioned and most prediction models for BPD only hold limited value for clinical use (8–10). Likewise, contemporary changes in the perinatal management of infants, such as the use of high-flow nasal cannula and less aggressive neonatal resuscitation, limit the application of prior definitions, and may result in further misclassification of the disease (11).

Unresolved as of today is the presumptive presence of different disease endotypes with variable contributions of airway pathology, matrix remodeling and pulmonary vascular disease, all covered by the umbrella term BPD.

As clinical understanding of the ever-changing picture of BPD is limited, conceptual insights derived from careful clinical observations and validated scientific findings in human babies

and preclinical animal models were and are needed to improve clinical standards. As a consequence, the use of antenatal steroids and postnatal surfactant have become routine and resuscitation has become gentler, especially with the previous imperative to get the baby fully oxygenated immediately after birth having been removed. The use of supplemental oxygen to maintain oxygen saturation in an evidence based low-normal range (12)—depending on lung vascular and extrapulmonary complications—is now known to be important as is the avoidance of unnecessarily high oxygen concentrations even for short periods of time. In addition to the reduction or prevention of oxygen toxicity, ventilation regimens have been profoundly revised with lower ventilation pressures and higher ventilation rates than were applied in the early years of neonatology (13, 14).

As important for minimizing the risk of sustained lung injury is the controlling of postnatal infections and the optimization of fluid balance (15, 16) and nutrition while preventing fluid overload and normalizing somatic growth. Prenatal risk factors including intra-amniotic infections and growth retardation are known to contribute to disease development in the structurally and functionally immature lung, but are—despite improved treatment regimen to initially support lung function and potentially maturation (17, 18)—still poorly controlled as of today and long-term benefits of existing therapies remain unclear.

Identification of the risk factors outlined above has helped to inform concepts of pre- and postnatal management although many challenges are only partially understood and therefore still unmet. Inevitable risks for BPD development remain including the higher risk for males. As both experimental and clinical studies revealed, other genetically determined risk factors are important, and an initial report has highlighted the involvement of a multitude of genes implicated in BPD (19).

Similarly challenging, new therapeutic developments that have been and will be highly beneficial for babies and their families, but are—as is well-known—accompanied by iatrogenic complications and change the pattern of disease in survivors. Two examples are the use of systemic corticosteroids in pre- and postnatal treatment regimens (17, 20, 21) and the careful consideration of potentially longer periods of oxygen exposure (22) as a tradeoff for a reduced period of invasive ventilation.

Nonetheless, even if neonatal practice was perfect, being born preterm is enough to lead to long term respiratory consequences, independent of any iatrogenic complications (23, 24).

In parallel with the change in the nature of BPD, tools for investigation and monitoring have become increasingly sophisticated. These include infant and pre-school lung function, including sensitive tests such as multi-breath washout, and imaging, initially with high-resolution computed tomography, and now increasingly with magnetic resonance imaging. Next to the need for further refinement of structural assessment using advanced imaging technologies while avoiding radiation exposure or general anesthesia, unmet challenges include the lack of biomarkers allowing for early disease detection and subsequent monitoring of progression. Here, multiomic technologies and systems biology may be of help including further sophisticated probing of potential BPD endotypes, that could allow individualized and maybe pathway specific treatment and monitoring approaches.

Adding to the complex disease picture, knowledge about co-morbidities is sparse but will—with increasing long-term survival—likely gain importance. The understanding of the late complications of BPD will inform neonatal practice, by analogy with cystic fibrosis, where long survival has led to the realization of the importance of detecting diabetes and bone disease in children and instituting preventive and treatment strategies. We have found out much about cardiovascular and metabolic morbidity and mortality in survivors; low first second forced expired volume (FEV_1) is a marker of increased risk in the normal term (25, 26), but also in BPD survivors (27, 28). Is our monitoring for diabetes and hyperlipidaemia adequate? These and other questions show that BPD is an illustration of the importance of not living in "developmental silos," but taking a whole life course view of traditional "pediatric" diseases.

In light of these considerations, a further challenge is engaging adult thoracic physicians in following these children up to determine the long-term consequences of BPD. Unfortunately, and in contrast to the tremendous investment in intensive care and the immediate aftermath, by and large respiratory follow up has not happened, despite the known respiratory morbidity in survivors (29). The survivors are thus often likely to be given inappropriate therapy such as treatment with inhaled corticosteroids for "asthma-like" phenotypes in preterm infants, despite the compelling evidence of a non-inflammatory airway phenotype in most BPD survivors with wheeze. Many will not attain normal spirometric values and will thus be at risk for being diagnosed with chronic obstructive pulmonary disease (COPD) (30). In addition, adult patient care often neglects early life events (31), increasing the risk for survivors of prematurity to be falsely combined under the COPD umbrella together with chronic smokers, although they may have a very different endotype leading to the end stage of a reduced FEV_1/forced vital capacity (FEV_1/FVC) ratio. This knowledge—together with the exchange of insights into potentially common pathways in neonatal and adult lung disease and the contribution to regeneration strategies informed by specialists in lung development—should lead to a fruitful exchange between experts and care givers. But as the face of the disease is changing, so a new generation of problems is on the way.

In face of all these achievements and remaining challenges, it becomes increasingly clear, that BPD is a dynamic disease that will be changing with neonatal practice and it would be unwise to assume that there will not be further important developments in the years ahead.

Understanding BPD therefore requires novel concepts stemming from clinically relevant experimental and translational approaches that undergo holistic, multi-layered evaluation to determine their utility for disease understanding and clinical care (see **Figure 1**). Showcasing the complexity of events is the need to understand cellular crosstalk in the

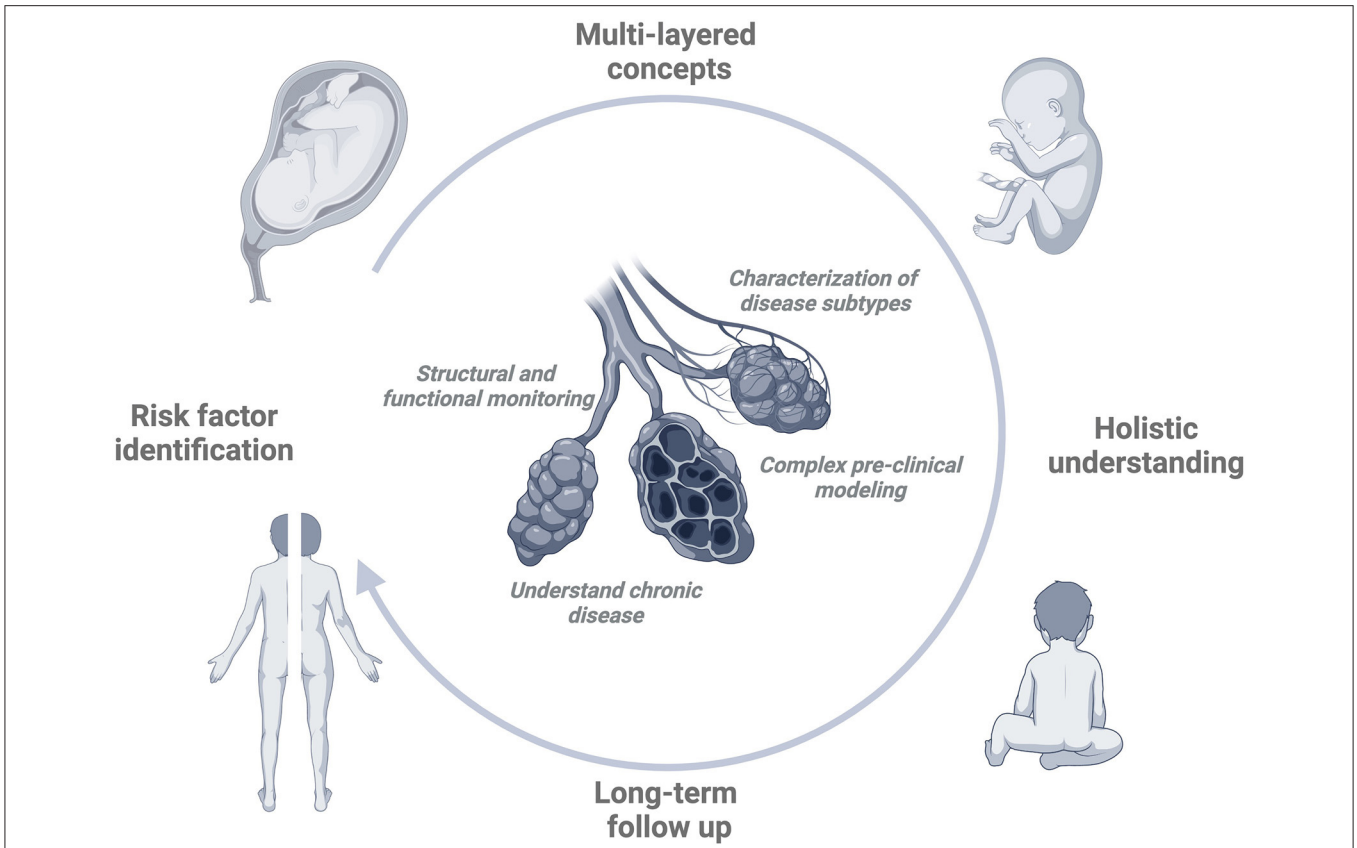

FIGURE 1 | BPD circle. In order to develop effective and comprehensive strategies to treat or prevent BPD, the identification and understanding of important pre- and postnatal contributors, i.e., risk factors is key. In order to generate a more holistic understanding of the disease, the development of multilayered pathophysiological concepts involving different areas of expertise will help us to move beyond over-simplified models of cause and consequence. In order to validate the relevance of proposed effects, insights obtained during long-term follow-up need to translate into knowledge about initial injury and risk factor impact. Bearing these goals in mind, the improvement of our understanding of the disease is supported by the development of diagnostic tools to better characterize lung structural changes and their functional consequences. The identification of (potential) disease 'subtypes' may allow us to understand more differentiated relationships between risk factor impact and clinical outcome. Complex, pre-clinical modeling is a prerequisite to drawing clinically relevant conclusions while considering the processes that mark the 'tipping point' of resolution to progression to chronic disease. Created with BioRender.com.

developing alveolar niche while considering the impact of the surrounding scaffold to gain insight into repair and regeneration capacities as well as mechanisms of progression to chronic disease. Premature ageing and the implications of senescence are poorly understood but likely play an important role in determining the outcomes. Questions that concern e.g., the mechanisms behind a "switch off" of a specific cellular developmental capacity in lung development and their later "switch on" in adolescence or adulthood have yet to be explored.

In order to inspire the development of novel, broader concepts, a comprehensive overview over the state of knowledge in the BPD field is needed covering ongoing research and clinical developments.

We have been privileged to work with a stellar group of authors and commentators to put this "BPD Research Topic" together. The content includes a comprehensive overview about available (and feasible) animal models to unravel disease mechanisms and perform preclinical studies (32, 33), insight into critical cell populations, the lung matrix and their complex interaction as determinators of the pulmonary landscape (2), and models of the nature and causation of lung injury reviewing the most critical pathophysiologic mechanisms (34, 35). Other manuscripts reflect on the role of new diagnostic and treatment concepts (36–39).

By combining specialist chapters with overview commentaries—often from authors who are experts in other fields of lung disease—we have furthermore aimed at giving a broader context to BPD with an "outside" view complementing the perspective from "within the NICU" (24).

We believe that the Research Topic on bronchopulmonary dysplasia provides a rounded and comprehensive picture of a disease that significantly determines quality of survival throughout the life course in one of the largest pediatric patient groups. It should paradigm for understating long-term consequences of early (multiple hit) injury.

AUTHOR CONTRIBUTIONS

AB and AH contributed as editors to the Research Topic on bronchopulmonary dysplasia by conceptualizing and supervising the content, inviting the contributing authors, and by writing the Editorial. Both authors contributed to the article and approved the submitted version.

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A breath of fresh air on the mesenchyme: impact of impaired mesenchymal development on the pathogenesis of bronchopulmonary dysplasia

*Cho-Ming Chao [1,2,3], Elie El Agha [2,3], Caterina Tiozzo [4], Parviz Minoo [5] and Saverio Bellusci [2,3,6,7]**

[1] Department of General Pediatrics and Neonatology, University Children's Hospital Giessen, Giessen, Germany, [2] Department of Internal Medicine II, Universities of Giessen and Marburg Lung Center, Giessen, Germany, [3] Member of the German Center for Lung Research (DZL), Giessen, Germany, [4] Division of Neonatology, Department of Pediatrics, Columbia University, New York, NY, USA, [5] Division of Newborn Medicine, Department of Pediatrics, Children's Hospital Los Angeles, University of Southern California, Los Angeles, CA, USA, [6] Saban Research Institute, Childrens Hospital Los Angeles, University of Southern California, Los Angeles, CA, USA, [7] Kazan Federal University, Kazan, Russia

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Anne Hilgendorff, Helmholtz Zentrum München, Germany
Andrew Bush, Royal Brompton Hospital and Imperial College London, UK

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Paschalis Sideras, Biomedical Research Foundation Academy of Athens, Greece

***Correspondence:**
Saverio Bellusci, Department of Internal Medicine II, Universities of Giessen and Marburg Lung Center (UGMLC), Klinikstraße 36, Giessen, Hessen 35392, Germany
saverio.bellusci@innere.med.uni-giessen.de

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The early mouse embryonic lung, with its robust and apparently reproducible branching pattern, has always fascinated developmental biologists. They have extensively used this embryonic organ to decipher the role of mammalian orthologs of *Drosophila* genes in controlling the process of branching morphogenesis. During the early pseudoglandular stage, the embryonic lung is formed mostly of tubes that keep on branching. As the branching takes place, progenitor cells located in niches are also amplified and progressively differentiate along the proximo-distal and dorso-ventral axes of the lung. Such elaborate processes require coordinated interactions between signaling molecules arising from and acting on four functional domains: the epithelium, the endothelium, the mesenchyme, and the mesothelium. These interactions, quite well characterized in a relatively simple lung tubular structure remain elusive in the successive developmental and postnatal phases of lung development. In particular, a better understanding of the process underlying the formation of secondary septa, key structural units characteristic of the alveologenesis phase, is still missing. This structure is critical for the formation of a mature lung as it allows the subdivision of saccules in the early neonatal lung into alveoli, thereby considerably expanding the respiratory surface. Interruption of alveologenesis in preterm neonates underlies the pathogenesis of chronic neonatal lung disease known as bronchopulmonary dysplasia. *De novo* formation of secondary septae appears also to be the limiting factor for lung regeneration in human patients with emphysema. In this review, we will therefore focus on what is known in terms of interactions between the different lung compartments and discuss the current understanding of mesenchymal cell lineage formation in the lung, focusing on secondary septae formation.

Keywords: lung development, alveologenesis, bronchopulmonary dysplasia, epithelial–mesenchymal interaction, endothelial–mesenchymal interaction, secondary septae formation

Bronchopulmonary Dysplasia is Characterized by Impaired Alveologenesis

Bronchopulmonary dysplasia (BPD) is a chronic lung disease of prematurely born infants and remains a leading cause of morbidity and mortality. Currently, there is no curative therapy available. Based on the severity-based definition of BPD (inclusion of infants with mild BPD) 68% of premature infants born with a gestational age (GA) $\leq$28 weeks develop BPD (1–3). The risk of developing BPD correlates inversely with the GA and birth weight (BW) (4). Since premature infants (24–28 weeks of gestation) are born with a lung, which is in the canalicular or saccular stages of development, the lung structure (characterized by thickened airspace walls and surfactant deficiency) is therefore not adequate to provide sufficient ventilation and gas exchange. Thus, mechanical ventilation and high-oxygen concentration are often necessary at birth. Barotrauma induced by mechanical ventilation as well as oxygen toxicity and inflammation are major contributing factors responsible for the pulmonary damages in the morphological and functional immature lung. In addition, some studies have suggested a strong genetic component in BPD (5). For example, using genome-wide association study, it has been shown that polymorphisms (SNPs) in *MMP16* and *SPOCK2* might be associated with BPD (6). Due to remarkable advances in the management and therapy (e.g., gentle ventilation, restricted oxygen supplementation, antenatal steroids, and exogenous surfactant use) survival rate for premature infants has increased over the last decades. These advances in treatment have changed the histological characteristics of what is now called the old BPD since it was first described by Northway in 1967. The "old" BPD was mostly an airway disease characterized by interstitial fibrosis and squamous metaplasia of airways. The prominent histological findings in the lungs of "new" BPD are simplification of alveolar formation (fewer and larger alveoli) and dysmorphic pulmonary microvasculature (7, 8). Pulmonary hypertension is also a common complication in infants with BPD, resulting in high mortality (9). According to these findings, the "new" BPD is considered as a consequence of the premature lung interrupted in its development by postnatal lung injury leading to the growth arrest of the lung in the canalicular/saccular phase of normal lung development. BPD, as a chronic lung disease, leads to long-term morbidity (e.g., pulmonary infection, neurodevelopmental impairment) affecting quality of life during childhood and in some severely affected patients even into adulthood. Treatment for BPD represents a considerable health care burden (10–12).

The mechanisms responsible for alveolar simplification in BPD remain understudied and poorly understood. However, autopsy samples from premature infants from pre- and post-surfactant era, who died from BPD consistently showed abnormalities in the mesenchyme (interstitial fibrosis and dysmorphic microvasculature). In the new BPD, there is clear evidence for decreased number of secondary septae, a derivative of the lung mesoderm. Furthermore, animal models mimicking the premature lung and the risk factors for BPD provide more evidence that indeed the mesenchyme plays a pivotal role in late lung development/alveologenesis and therefore in BPD. This review will summarize the current understanding of the impaired mesenchymal compartment of the BPD lungs, with a focus on mesenchymal–endothelial and mesenchymal–epithelial crosstalk known to contribute to disease pathogenesis.

Normal Lung Development in Human and Mouse

In human and mouse, the lung arises from two germ layers: the gut endoderm gives rise to the lung epithelium and the splanchnic mesoderm is the origin of the lung mesenchyme. The human lung consists of three lobes on the right and two lobes on the left side; in mice four lobes form on the right (cranial, medial, caudal, and accessory lobe) and one on the left. Compared to the 12 airway generations observed in mice, human lungs comprise 23 airway generations.

In humans, lung development arises from the laryngo-tracheal groove and starts at week 4 of gestation as an outgrowth from the ventral wall of the caudal primitive foregut. During the further growth of the lung, the prospective trachea separates from the foregut by the formation of the so-called tracheo-esophageal septum. At the most distal part of the tracheal tube, two buds that will form the right and left primary bronchial buds appear. These primary buds are further ramified to form three secondary bronchial buds on the right and two secondary bronchial buds on the left side. These buds are the origin of the five lobes in the mature lung (13).

In mice, at embryonic day 8 (E8), signaling molecules and growth factors (e.g., Fgf1, Fgf2) emanate from the cardiac mesoderm and specify the prospective lung field in the primitive foregut endoderm, which is positive for the transcription factor *Nkx2.1* (or *Ttf1*). These pre-lung epithelial progenitor cells represent the earliest and most likely the most pluripotent epithelial cells for the lung. At E9.5, the ventral foregut endoderm evaginates and elongates caudally dividing into two buds that form the prospective trachea and the first generation of bronchi (main bronchi). The process of lung development (human and mouse) has been divided into four distinct histological phases: pseudoglandular, canalicular, saccular, and alveolar (**Figure 1**).

During the pseudoglandular stage (human: week 4–17; mouse: E9.5–E16.5), the process of branching morphogenesis generates the basic tree-like structure of the lung including the conducting airways and the numerous terminal bronchioles surrounded by thick mesenchyme. Concurrently, epithelial cell progenitors undergo differentiation to give rise to basal, neuroendocrine, ciliated, and secretory cells. The mesodermal lung compartment serves as progenitors for the smooth muscle, lymphatic, endothelial, nerve, and chondrocytic cells.

In the subsequent canalicular stage (human: week 17–26; mouse: E16.5–E17.5), the lung undergoes further subdivision of the respiratory bronchioles accompanied by thinning of the surrounding mesenchyme and the massive formation of capillaries. For the first time during development, a primitive respiratory epithelium competent of gas exchange is formed by differentiation of distal lung epithelial progenitors. Recently, it has been shown that type I and type II alveolar epithelial cells (AEC I and II) emerge from a common alveolar bipotential progenitor (14). In mice, interstitial fibroblasts containing cytoplasmic lipid droplets (so called lipofibroblast, LIF) emerge in

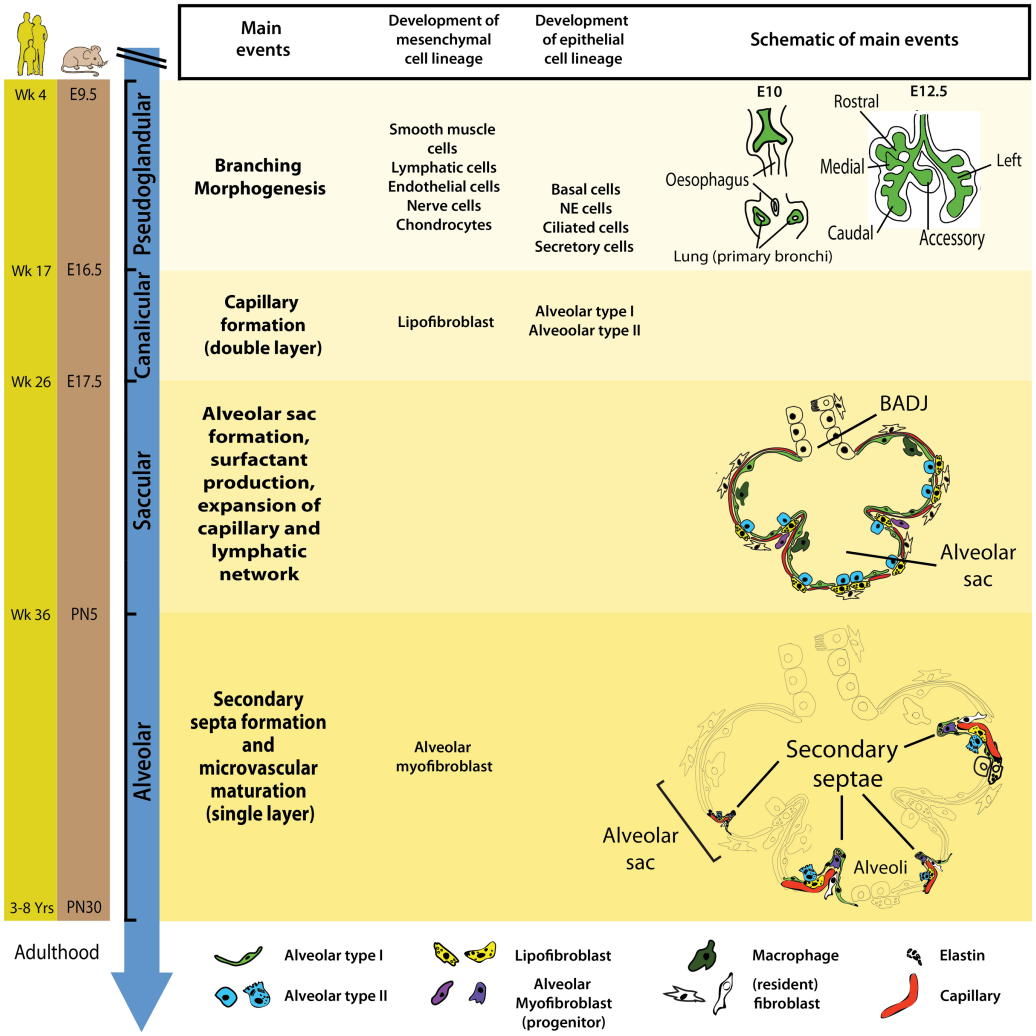

FIGURE 1 | Timeline and stages of lung development in mice and humans. Lung development starts with the specification of the lung domain in the foregut endoderm followed by the formation of primary lung buds. These buds will later give rise to the respiratory tree via the process of branching morphogenesis. The latter is a characteristic of the pseudoglandular stage of lung development. Most epithelial and mesenchymal cell types start to form during the pseudoglandular stage. The canalicular stage is characterized by blood capillary formation and the appearance of AECI/II. During the saccular stage, primitive alveoli (sac-like structures) start to form and this is accompanied by surfactant production and the expansion of capillary and lymphatic networks. The alveolar stage of lung developments starts *in utero* in humans whereas in mice, it starts postnatally. Wk, week; E, embryonic; PN, postnatal; NE, neuroendocrine.

the mesenchyme. Additionally, this is the earliest time point of pregnancy (23–24 weeks of gestation) where a preterm infant can be born with a chance to survive. Those who died from BPD showed pathologic characteristics of the lung (interstitial fibrosis and dysmorphic microvasculature) similar to the morphology of the immature lung at this developmental stage thus reinforcing the concept that BPD results from interruption of normal lung development by deleterious environmental events. The introduction of antenatal steroids treatment and exogenous surfactant supplementation drastically increased survival of premature infants born at this stage (15).

The saccular stage of lung development occurs approximately between 26 and 36 weeks of gestation (mouse: E17.5–PN5). This stage is characterized by the formation of alveolar sacs, surfactant production, and thinning of the mesenchyme to facilitate gas exchange. Kresch demonstrated that the thinning of the mesenchyme results from apoptosis of mesenchymal cells (16). Furthermore, the capillary and lymphatic networks also expand in the saccular stage of lung development.

The last stage of lung development is termed alveolar stage (human: ~36 weeks to 8 years; mouse: PN5–PN30). During this stage, the alveolar surface area increases massively at the expense of the mesenchyme through subdividing the alveolar sacs (also called primitive alveoli) into mature alveoli by a process termed alveolarization (or alveologenesis) (**Figure 2**). This process starts with the deposition of elastin in primary septae (wall of alveolar sacs) and subsequently secondary septae emerge at the place of elastin and elongate toward the alveolar sac airspace to subdivide

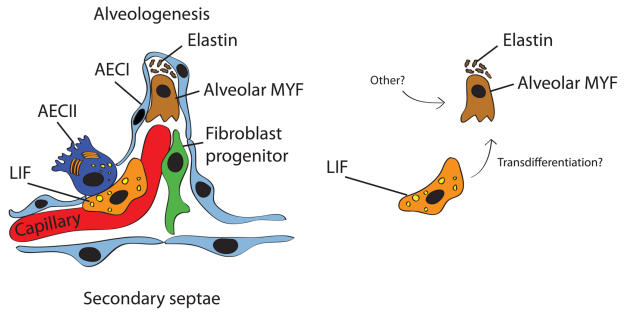

FIGURE 2 | Schematic representation of the secondary septum during alveologenesis. Most of the alveolar surface is occupied by AECI (gas exchange) whereas a minor surface is occupied by AECII (surfactant production). The alveolar wall consists of the blood capillary, LIF, resident fibroblast progenitor, alveolar MYF, and ECM (mostly elastin). It has been proposed that alveolar MYF can originate from LIF (right panel) but this concept needs further validation.

it into the smallest respiratory units of the lung – the mature alveoli. Importantly, concomitant with this process, primary septae, still containing a double layer of capillaries, become thinner and a single capillary network emerges allowing more efficient gas exchange (microvascular maturation). The bulk of alveolarization takes place during the first 6 months after birth in humans (mouse: PN5–PN15) (17). The alveolar myofibroblast (MYF), localized in the mesenchyme at the tip of the emerging secondary septae, is the cell responsible for secondary septae formation. A more detailed description of this mesenchymal cell lineage will be provided in the following sections.

In summary, the lung is a complex ramified organ that develops through continuous and elaborate interaction among the epithelium, mesenchyme, mesothelium, and endothelium. During this process, an intricate signaling network controls the amplification, proliferation, migration, and differentiation of diverse progenitor cells to populate these different compartments. Importantly, most of the epithelial and mesenchymal cell types in the lung are formed during the late pseudoglandular stage (E13.5–E16.6). This means that any deleterious factors present prenatally (such as inflammations due to chorioamnionitis) or postnatally (such as barotrauma injury and subsequent inflammation due to oxygen or mechanical ventilation), interfering with normal lung development at that time, could lead to impaired pulmonary function postnatally. Since preterm infants who die from BPD commonly display abnormal mesenchyme, a better understanding of aberrant signaling pathways in the lung mesenchyme of BPD lungs is important for improving the existing, and may facilitate the development of new preventive and curative therapies. In the next section, the current knowledge, mostly obtained from animal models of BPD, about abnormalities occurring in the lung mesenchyme will be reviewed.

The Embryonic Lung Mesenchyme

During the pseudoglandular stage of lung development (~E13.5), the distal lung bud is composed of three morphologically distinguishable layers: the mesothelium (outer layer), the mesenchyme (middle layer), and the epithelium (inner layer). The mesenchyme can be further divided into two domains, the submesothelial mesenchyme (SMM) and the subepithelial mesenchyme (SEM). Whereas mesenchymal cells constituting the SEM display high density and circumferential orientation, those of the SMM display low density and organization. Lineage-tracing experiments have identified markers for some mesenchymal progenitors such as Wnt2/Gli1/Isl1 (originating from the heart and invading the lung), Ret, Pdgfrα, Vegfr2, Prox1, and Fgf10 (18–20). Progenitors in these two compartments give rise to various cell types such as airway smooth muscle cells (ASMCs), vascular smooth muscle cells (VSMCs), resident mesenchymal stem cells (MSCs), LIFs, endothelial cells, chondrocytes, nerve cells, alveolar MYFs, lymphatic cells, and others. Mesenchymal progenitor cells are believed to play important roles not only in development but also in homeostasis and regeneration after injury.

Epithelial–Mesenchymal Crosstalk in Normal Lung Development and BPD

During development, the lung is formed through an elaborated epithelial–mesenchymal crosstalk that drives lung specification, budding, and branching. Signaling molecules like fibroblast growth factors (Fgf), Wnt (wingless and int), Sonic hedgehog (Shh), and bone morphogenetic proteins (Bmp) are key ligands initiating the pulmonary cell fate and specifying the early lung domain at the ventral foregut endoderm (21). So far, the most convincing evidence for epithelial–mesenchymal interactions during lung development came from recombination studies where distal lung mesenchyme, grafted on the tracheal epithelium led to ectopic budding accompanied by expression of surfactant protein C as a distal epithelial marker (22–24).

The mammalian Fgf family consists of 22 members subdivided in 7 subfamilies, based on phylogenetic as well as gene loci analyses (25). Fgfs acts in a paracrine, endocrine, or intracrine fashion and have diverse biological activities during embryonic organogenesis. These growth factors act via seven main receptors (Fgfrs 1b, 1c, 2b, 2c, 3b, 3c, and 4), exhibiting different ligand-binding specificity. The Fgf receptors are encoded by four *Fgfr* genes (*Fgfr1–Fgfr4*), which undergo alternative splicing to produce the different isoforms. Each receptor comprises an extracellular ligand-binding domain with three immunoglobulin-like loops (D I, D II, D III), a transmembrane domain and an intracellular tyrosine kinase domain. Human diseases involving gain or loss of function mutations have been described. For example, loss of function of *FGF3* causes deafness, heterozygous loss of function of *FGF10* results in lacrimo-auriculo-dento-digital syndrome (LADD syndrome), *FGF10* haploinsufficiency is also associated with chronic obstructive pulmonary disease and *FGF23* gain of function leads to autosomal dominant hypophosphataemic rickets (26–29). During early (E12.5) embryonic mouse lung development, Fgf9 and Fgf10 have been shown to play an important role in branching morphogenesis and the associated differentiation of the epithelium and mesenchyme. Fgf9 is expressed in the mesothelium and the epithelium and acts through Fgfr2c- and Fgfr1c-expressing cells in the mesenchyme to maintain Fgf10 expression as well as mesenchymal progenitors proliferative and

undifferentiated (30). It also can signal directly to the epithelium to promote epithelial branching by induction of *Dkk1* expression and inhibition of Wnt signaling (31). Fgf10 is a diffusible key molecule orchestrating branching morphogenesis during early lung development in mice (32, 33) but the exact mechanism of action remains unknown. During the early pseudoglandular stage, Fgf10 is secreted by cells located adjacent to the mesothelium in the distal mesenchyme and signals in a paracrine manner mainly through fibroblast growth factor receptor 2-IIIb (Fgfr2b) expressed on epithelial cells. Fgf10 has a high affinity for heparan sulfate and is therefore unlikely to diffuse over a long distance. Instead, Fgf10 promotes outgrowth of the distal epithelium via a chemotactic mechanism. Several studies using transgenic mouse lines that display abnormal Fgf10/Fgfr2b signaling confirmed the importance of this pathway (**Table 1**). *Fgf10* and *Fgfr2b* knockout pups display similar phenotypes. The mutant pups die shortly after birth due to lung agenesis and multiple organ agenesis/defects (salivary gland, limb, inner ear, teeth, skin, pancreas, kidney, thyroid, pituitary gland, mammary gland) (34–38).

In order to identify epithelial-specific gene expressions mediated by recombinant human FGF10 during bud morphogenesis, Lu and colleagues (39) used mesenchyme-free epithelium in culture. By using microarray analysis, they identified a panel of transcriptional *Fgf10* targets, which are associated with cell rearrangement, migration, inflammatory processes, lipid metabolism, cell cycle, and tumor invasion. Interestingly, the authors did not observe a remarkable induction of genes responsible for proliferation. Moreover, Fgf10 is proposed to control the angle of the mitotic spindle in distal epithelial cells during development. Thus, Tang et al. argued that Fgf10 signals via a Ras-regulated Erk1/2 signaling pathway to shape the lung tube (40). Fgf10 is also critical for the amplification of distal epithelial cell progenitors and for the formation of multiple mesenchymal lineages during lung development. Hypomorphic $Fgf10^{lacZ/-}$ pups expressing ~20% *Fgf10* compared to wild type (WT) died within 24–48 h after birth due to lung defects, which included decreased branching, thickened primary septae, and vascular abnormalities with intrapulmonary hemorrhages. At the cellular level, *Fgf10* deficiency led to decrease in Nkx2.1 and Sftpb-expressing cells, suggesting that adequate *Fgf10* expression level is critical for the amplification of epithelial progenitors. Apart from the epithelium, constitutive decrease in *Fgf10* expression also affects mesenchymal cell lineages as *Pecam* and α*Sma*-positive cells are also diminished (41). Interestingly, recent experiments conducted in our lab to investigate the impact of *Fgf10* levels on lung function demonstrate that even a 50% decrease in *Fgf10* expression (*Fgf10* heterozygous pups) leads to changes in the expression of genes relevant for lung development such as *Epcam, Sftpc*, *Fgfr2b*, *Tgf*-β, and *Collagen*. Additionally, *Fgf10* heterozygous neonatal mice survive and do not display any obvious phenotypic differences compared to WT mice. However, when exposed to hyperoxia between PN0 and PN8 to trigger lung injury and mimic some of the clinical manifestations of BPD (impaired alveologenesis and inflammation), *Fgf10*-deficient pups display drastically increased mortality compared to WT controls. Further analysis indicates that under physiological conditions, *Fgf10*-deficient mice already show structural abnormalities during embryonic lung development supporting that *Fgf10*-deficient pups carry congenital defects. These findings suggest that *Fgf10*-deficient lung epithelium is more susceptible to oxygen toxicity and does not undergo normal repair after injury

TABLE 1 | Overview of proteins that are known to be involved in alveologenesis.

Protein name	Origin	Localization/ targets	Function in alveologenesis	Alterations in BPD	Alterations in animal model of BPD	Effect of genetic modulation in the animal model
Elastin	Alveolar myofibroblast	Tip of growing secondary septae	Secondary septae formation (tips)	Increased and disorganized in saccular walls (66, 67)	Decreased in hyperoxia (133)	KO: inhibited alveolarization (87)
Pdgfa	Epithelial cells, macrophages	Pdgfrα-expressing cells (ASMC, alv. MYF, LIF)	Chemotactic attractant for fibroblasts (134)	Not known	Delayed in hyperoxia (135)	KO: inhibited alveolarization (93, 94)
Fgf10	Mesenchymal cells located in SMM	Distal epithelial cells expressing Fgfr2b	Under investigation	Decreased (75)	Decreased in LPS-model (76)	KO: lung agenesis Partial deficiency: delayed/disturbed lung branching (41)
Tgf-β/ Tgf-β1	Epithelial cells	Epithelial and mesenchymal cells	Modulation of cell survival, differentiation and ECM (Elastin) deposition (136, 137)	Increased in tracheal aspirate (138)	Increased in hyperoxia (139, 140)	Overexpression: inhibition of branching morphogenesis and alveolarization (141) Inhibition: attenuated hyperoxia-induced hypoalveolarization (140)
Vegf	Epithelial (during embryonic development also in mesenchymal cells)	Endothelial cells (Vegfr1/2)	Stimulation of endothelial cells for angio-/vasculogenesis (essential for alveolarization)	Decreased (8, 127)	Decreased in hyperoxia (125); (126)	Inhibition: hypoalveolarization (142, 143)

(Chao and Bellusci, in preparation). Additionally, recent studies suggest that Fgf10 may control basal cell density in the tracheal epithelium (42–45). This is not surprising as it has already been previously shown, by our group and others, that Fgf10 is part of the stem cell niche in the lung (20, 46, 47).

Wnt (Wnt2, Wnt2b) ligands are expressed in the mesenchyme and are important for lung domain specification of the foregut endoderm from E9.0 to E10.5. Wnt signaling is also essential for the proximo-distal patterning of the epithelium during embryonic lung development. Genetic deletion of *Wnt2/2b* or β-*catenin* leads to lung agenesis due to loss of *Nkx2.1* (48, 49). *Wnt2* null mice display lung hypoplasia and abnormal development of ASMCs (50). Furthermore, Mucenski and colleagues demonstrated, by using *Spc-rtTA;tet(O)Cre* double transgenic mice, that loss of function of β-*catenin* in the distal lung epithelium leads to the inhibition of distal airway formation (51). The authors showed an opposite phenotype by inducing gain of function of β-*catenin* signaling (52). The absence of *Wnt7b* results in a phenotype similar to *Wnt2* null mice (53) but a combination of *Wnt7b* and *Wnt2* loss of function leads to a more severe phenotype with decreased branching and abnormal distal endoderm patterning (54). The constitutive deletion of *Wnt5a* – a non-canonical Wnt ligand expressed in the mesenchyme and the epithelium – leads to increased proliferation of the mesenchyme and the distal epithelium as well as disrupted lung maturation (55). β-*catenin* inactivation in the mesenchyme (*Dermo1-Cre* line) leads to abnormal mesenchyme development with disrupted amplification of ASMC progenitors and defects in angioblast differentiation (56). Kumar and colleagues demonstrated by using a clonal cell labeling approach that ASMC progenitors are located exclusively at the tip mesenchyme and that mesenchymal Wnt signaling is able to prime the stalk mesenchyme to form an ASMC progenitor pool at the tip (57).

Bmp4 is dynamically expressed in the endoderm and in the mesenchyme during early embryonic lung development (E11.5). It is also expressed at the distal epithelial buds and has been shown to be an inhibitor of Fgf10-induced chemotaxis in the epithelium. Bmp4 controls intraepithelial crosstalk to form ASMCs. It has been shown that *Fgf10* is able to upregulate *Bmp4* mRNA expression. *In vitro* experiments demonstrated that exogenous recombinant human BMP4 inhibits Fgf10-induced bud outgrowth, providing evidence that Bmp4 is acting downstream of Fgf10 to inhibit its signaling cascade (58–61).

Other Fgf10 inhibitors are Sonic hedgehog (Shh) and Sprouty homolog 2 (Spry2), both expressed in the epithelium of the outgrowing buds. Shh is a secreted growth factor that acts through its mesenchymal receptor Patched (Ptc) to induce mesenchymal cell proliferation and differentiation. In E11.5 lung explants, exogenous recombinant SHH is able to induce expression of mesenchymal markers (*Noggin*, *Acta2*, *Myosin*) (19, 62). Spry2 is an intracellular inhibitor of receptor tyrosine kinase signaling (63, 64); (32). Using *in vitro* approaches, it has been shown that *Spry2* reduction leads to increased epithelial branching and vice versa.

Apart from its important role in development, the mesenchyme is crucial in disease pathogenesis. Indeed, it has been reproducibly shown that the lung mesenchyme in preterm infants dying from BPD includes interstitial fibrosis and thickening with increased total collagen content (65–67). Similar findings were obtained in diverse animal models (rat, mice, baboon) recapitulating the conditions of preterm infants after birth (mechanical ventilation, oxygen supplementation, exogenous surfactant) leading to a human BPD-like phenotype (68–72). These pathological changes in the lung mesenchyme in BPD strengthen the concept that alveolarization depends on an intact and normally developed mesenchyme. Several studies using animal models of BPD to identify molecules located in the altered lung mesenchyme contributed to our understanding of disease pathogenesis. Some of them will be reviewed in the following section.

One of the major causes of BPD is believed to be inflammation. Inflammation is caused prenatally by chorioamnionitis and postnatally by mechanical stretch (ventilation), oxygen toxicity, as well as infection. Emerging evidence gained from *in vitro* and *in vivo* studies support this hypothesis (73–77). For example, it has been shown that lipopolysaccharides (LPS from *Escherichia coli*) inhibit branching morphogenesis *in vitro* (73). Blackwell et al. published similar results using activated resident macrophages to inhibit epithelial branching. The proposed mechanism is that LPS activates nuclear factor kappa beta (NF-kappa B), which is then accompanied by increased expression of *interleukin-1beta* (*IL-1*β) and *tumor necrosis factor-a* (*TNF-a*) in resident macrophages (74). This branching inhibitory effect caused by macrophage-mediated inflammation has been confirmed by a macrophage-depletion study in the lung. Benjamin et al. explained this inhibitory effect by linking Fgf10 signaling with inflammatory signals. Using *in vitro* experiments, they demonstrated that NF-kappa B, IL-1β, and TNF-*a* are capable of reducing *Fgf10* expression in LPS-treated primary mesenchymal cells. The mechanism involved activation of toll-like receptors 2 and 4 (TLR2/4). The authors showed that FGF10-positive cells were decreased in lung samples of premature infants who died from BPD (75).

Tgf-β1 has been demonstrated to induce epithelial–mesenchymal transition (EMT) of AEC to MYF-like cells leading to extracellular matrix (ECM) deposition and thereby contributing to fibrosis and destruction of alveolar structure (78–80) (see also **Table 1**). Endogenous nitric oxide is proposed to attenuate EMT in AECs in an *in vitro* approach using primary culture of AEC II (81).

As previously mentioned, the alveologenesis phase leads to a dramatic increase in alveolar surface, which is essential for gas exchange. The current consensus is that this process is interrupted by exogenous deleterious factors leading to simplification of alveoli in BPD. Many studies confirmed that the alveolar MYF, located in the mesenchyme, is the unique cell type responsible for secondary septae formation. During alveologenesis, the alveolar myofibroblast is characterized by expression of alpha-smooth-muscle-actin (αSMA or Acta2) compared to other mesenchymal fibroblast population. By deposition of elastin and collagen, the alveolar myofibroblast initiates the process of secondary septation (82, 83). Both elastin and alveolar myofibroblast have been shown to be critical for secondary septae formation (83, 84). Expression of tropoelastin starts in the pseudoglandular stage of lung development and reaches the highest level during the alveolar stage (85, 86). The strongest evidence so far showing the importance of elastin for secondary septae formation came

from the *Elastin*-knock-out mice that reveal a complete failure of alveologenesis leading to an emphysematous-like phenotype (87, 88) (**Table 1**). Interestingly, both hyperoxia and mechanical ventilation lead to increased expression of Elastin (89–91). Fgfr3 and Fgfr4 have been shown to direct alveologenesis in the murine lung by controlling elastogenesis (92). By using mice homozygous for *Pdgfa*-null allele, Boström and colleagues demonstrated failed alveolar formation due to loss of alveolar myofibroblasts and consequent loss of Elastin fibers (93, 94). Likewise, blocking antibody against Pdgfrα in newborn mice (PN1–PN7) led to aberrant Elastin fiber deposition and impaired alveolar septation, resulting in long-term failure in alveologenesis that lasted into adulthood. Pdgfa is expressed in the epithelium and targets its receptor (Pdgfrα) on mesenchymal cells such as alveolar myofibroblast and LIF (**Table 1**). Given the many mesenchymal targets of Pdgfa, it is not clear whether the impact of Pdgfa or Pdgfrα deletion on myofibroblast formation is via a direct effect of Pdgfa on alveolar myofibroblasts (and or alveolar myofibroblast progenitors) or indirectly via Pdgfa action on other targets (ASMCs and LIF). Gain and loss of function for Pdgfa/Pdgfrα signaling using cell autonomous-based approaches in specific lineages should be carried out in the future to sort out these issues.

Interestingly, increased levels of *Fgf* signaling in the mesenchyme also leads to arrested development of terminal airways accompanied by reduced Elastin deposition (95). The authors achieved this condition by taking advantage of *Fgfr2c*$^{+/\Delta}$ mice that develop an autocrine Fgf10–Fgfr2b signaling loop in the mesenchyme due to a splicing switch, resulting in the ectopic expression of *Fgfr2b* instead of *Fgfr2c*. The proposed mechanism of action is that mesenchymal *Fgf* signaling suppresses the differentiation of alveolar myofibroblast progenitors. Furthermore, the blockade of Fgfr2b ligands in the lung from E14.5 to E18.5 by overexpression of a soluble dominant negative receptor of *Fgfr2b* (*Sftpc-rtTA/+;tetOsolFgfr2b/+*) blocking all Fgfr2b ligands also leads to arrest in secondary septae formation and alveolar simplification (96) suggesting that Fgfr2b ligands, during this time period, are also important for the formation of alveolar myofibroblasts. Subsequent treatment with retinoic acid (RA, biologically active derivative of vitamin A) induced re-alveolarization and was accompanied by increased *Pdgfra*-positive cells and decreased α*Sma/Acta2*-positive cells. Concurrent induction of the dominant negative *Fgfr2b* in these experimental conditions is able to prevent the RA-mediated alveolar regeneration. These data suggest that re-alveolarization is dependent on Fgfr2b ligands. Furthermore, the authors proposed a conceptual model that alveolar myofibroblasts (α*Sma/Acta2*-positive) arise from *Pdgfra*-positive LIF. Specific lineage-tracing studies targeting subsets of lung fibroblasts (e.g., *Adrp* for LIF) are needed to validate this model. Chen and colleagues also demonstrated that Fgfr2b ligands are necessary for alveolar myofibroblast formation during compensatory lung growth after pneumonectomy (97). However, the blockade of Fgfr2b ligands by soluble Fgfr2b (decoy receptor) postnatally during alveolarization does not impair alveologenesis in mice. Recently, it has been shown that reduced *Pdgfra* expression is a primary feature of human BPD. The authors showed decreased mRNA and protein expression of *PDGFR*-α and *PDGFR*-β in MSCs isolated from tracheal aspirates of premature neonates with BPD. Similarly, lungs of infants dying from BPD display less PDGFRα-positive cells in the alveolar septae. These findings were confirmed using a BPD mouse model exposed to hyperoxia (75% oxygen) for 14 days (98).

The LIF remains a poorly characterized lipid-containing interstitial cell located in the mesenchyme in close proximity to AEC II. LIFs, which accumulate lipid vacuoles (83, 99), are abundant in the early postnatal lung and regress significantly in number after alveolar septation. The presence of LIF in rodent lungs has been demonstrated extensively. However, whether LIF reside in adult human lung remains controversial (100, 101). Because of their close localization to AEC II, LIF have been proposed to interact with AEC II. Indeed, it has been shown convincingly that LIF are involved in the trafficking of lipids to the AEC II for surfactant production (102, 103). Apart from triglycerides, LIF also secrete leptin and retinoic acid, both important for surfactant production and alveolar septation (104, 105). On the other hand, AECII secrete parathyroid hormone-related protein (Pthrp) to signal through Pthrp receptor expressed on LIF to induce expression of adipose differentiation-related protein (*Adrp*) via peroxisome proliferator-activated receptor gamma (Pparg) pathway (**Figure 3**). The current consensus is that this signaling pathway is essential for the maintenance of the LIF phenotype as well as for regulation of surfactant production (104, 106–108). By performing co-culture experiments it has been proposed recently that LIF constitute a niche for AECII cells postnatally. Co-culture of LIF with AECII cells allows the formation of alveolospheres (109).

The contribution of LIF to lung regeneration and structural maintenance in later phases of life is currently unknown. Several lung injury models including cigarette-smoke exposure induce the transdifferentiation of LIF to αSma/Acta2^{+} MYF *in vitro*. These αSma/Acta2^{+} MYF are highly proliferative and express high levels of collagen (110). For this reason, it has been proposed that LIF are progenitors for alveolar MYF (**Figure 2**). However, an alternative and more plausible possibility is that LIF give rise to the activated MYF. Activated MYF, unlike the alveolar MYF, is involved in pathological situations and is responsible for fibrosis. Supporting this possibility, we have recently shown that during alveologenesis, *Fgf10*-positive cells give rise to LIF

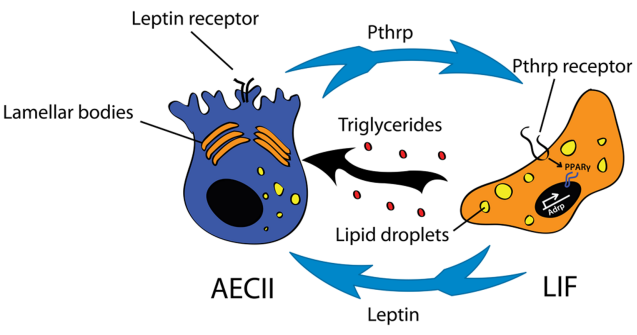

FIGURE 3 | Interaction between type II alveolar epithelial cells (AEC II) and lipofibroblasts (LIF) for surfactant production. The Pthrp (parathyroid hormone-related protein)/Pparg (peroxisome proliferator-activated receptor gamma) axis is important for LIF formation and maintenance. LIF secrete triglycerides and leptin that are essential for surfactant production.

rather than alveolar MYF and during adult life, a subpopulation of *Fgf10*-expressing cells represents a pool of resident MSCs ($Cd45^-$ $Cd31^-$ $Sca\text{-}1^+$) (20). In addition, the LIF-to-"activated MYF" transdifferentiation would translate indeed into loss of pulmonary integrity by smoke *in vivo*. Such transdifferentiation can be prevented and reversed *in vitro* using Pparg agonists such as rosiglitazone (111). However, it remains unclear whether such transdifferentiation occurs *in vivo*. Of note, exposure of premature neonates to hyperoxia induces arrest of alveolar septation and thickened primary septae due to MYF hyperplasia and excessive ECM production. Therefore, LIFs are unlikely progenitors for alveolar MYF. In the future, these results will have to be confirmed by lineage-tracing experiments in the context of injury using more specific knock-in lines to target the LIF and determine their fate.

Endothelial–Mesenchymal Crosstalk in Normal Lung Development and BPD

In parallel to branching morphogenesis during early embryonic lung development the lung vasculature begins to form in the mesenchyme at around E10.0 (112). This process involves angiogenesis and vasculogenesis. Angiogenesis occurs when preexisting endothelial cells sprout to form capillaries. In comparison, vasculogenesis is characterized by migration and differentiation of endothelial progenitor cells (or hemangioblasts) in the distal mesenchyme to form new blood vessels. DeMello and colleagues investigated the early fetal development of lung vasculature by employing light and transmission electron microscopy as well as vascular casts and scanning electron microscopy. They demonstrated three features of the lung vasculature occurring between E9.0 and E20.0 in mice: (1) angiogenesis occurs in the proximal (central) lung vasculature, (2) peripheral lung vessels are established by vasculogenesis, and (3) at E13.0/E14.0 the central and peripheral parts of lung vasculature begin to connect to each other via a lytic process (113, 114). Finally, the main event of microvascular maturation takes place during the alveolar stage of lung development where the transition from a double capillary network to a single capillary system within alveolar walls occurs.

Although BPD has long been regarded as an epithelial disease due to its emphysematous aspect, much emphasis has now been placed also on the role of the lung vasculature in this disease. The temporal–spatial proximity of lung vasculature development and branching morphogenesis suggests a close interaction between these two important structures via endothelial–epithelial tissue crosstalk. The better understanding of this crosstalk in development and disease condition might be highly relevant for future therapies. Vascular endothelial growth factor receptor 2 (Vegfr2 or Flk-1) is an early marker for endothelial progenitors located in the SEM (115, 116). However, it is not yet clear whether these progenitors arise from the mesothelium or the mesenchyme (117, 118). Progenitors for VSMCs are believed to arise from $Fgf10^+$ cells (20), $Wnt2^+$, $Gli1^+$ and $Isl1^+$ cells (coming from the second heart field) (18), $Pdgfrb^+$ cells (119), and mesothelial cells (117, 118).

During murine embryonic development, Vegfr2-positive cells receive the Vegfa signal from epithelial and mesenchymal cells until E14.5, after which *Vegfa* expression becomes restricted to the epithelium (120) (**Table 1**). Furthermore, it has been shown that Shh and Fgf9, secreted by the epithelium, are able to induce expression of *Vegfa* in the mesenchyme (121). Reciprocally, mesenchymally secreted Fgf10 leads to the upregulation of Vegf in the distal epithelium (122). In our previous work, we showed that treatment of embryonic lung explants with recombinant Vegfa not only upregulates *Vegfr2* in the mesenchyme but also induces branching of the epithelium (123). However, it is unclear whether the effect of Vegfa on epithelial branching is direct or indirect. This endothelial–epithelial tissue crosstalk has been extensively examined by using *in vitro* recombination studies (co-culture of epithelium and mesenchyme respectively and mesenchyme alone) as well as in *in vivo* lung agenesis model (β-*catenin* knockout) (112). Using an *in vivo* inducible decoy receptor of Vegfr1 (solubleVegfr1), Lazarus and colleagues demonstrated that Spry2 is upregulated in the epithelium upon inhibition of Vegfr1-mediated signaling, suggesting an inhibition of Fgf signaling (as mentioned before Spry2 is an inhibitor of Fgf10), which is essential for branching morphogenesis (124). Another link in the endothelial–epithelial crosstalk came from the *Pecam1*-deficient mice that display a failure in endothelial cell formation accompanied by simplified alveolarization (125).

During a pathological process, Vegfa has been found downregulated in preterm infants with BPD (8, 126, 127). Furthermore, Thebaud and colleagues demonstrated that *Vegf* and *Vegfr2* are decreased in the hyperoxia model of BPD in newborn rats and that adenoviral administration of VEGF improved alveolar architecture and promoted capillary formation (128, 129). Although the trophic and angiogenic potential of VEGF on the lung vasculature is known, the aforementioned study, and the studies from other groups, suggest that vascular growth serves as a driving force for alveolar growth and maturation, leading to improvement of lung structure, and promoting secondary septae formation. A recent report revealed the association of a *VEGF* polymorphism with BPD in Japanese preterm newborns (130).

Newborn mice that are hypomorphic for *Fgf10* also display reduced expression of *Vegfa* and *Pecam*. These mice suffer from an oversimplified lung with an abnormally developed lung vasculature (41). Interestingly, *Fgf10* expression is reduced in lungs from BPD patients (75). Whether the effect of mesenchyme-derived growth factors (such as Fgf10) on the lung endothelium is direct needs to be demonstrated. Another animal injury model demonstrating the importance of endothelial–epithelial interactions is the pneumonectomy model in mice. An inducible endothelium-specific deletion of *Vegfr2* and *Fgfr1* leads to reduction of Mmp14 secretion. Mmp14 is critical for expansion of epithelial progenitor cells during compensatory lung growth by unmasking Egfr ecto-domains. This was confirmed convincingly by rescue-experiments where EGF/MMP14 administration resulted in restored alveologenesis (131).

Conclusion

Although advances in pharmacotherapy and medical technology (e.g., gentle ventilation) have improved the management of premature infants, BPD remains the most common incurable chronic lung disease of infancy with considerable mortality and long-term morbidity. An unintended consequence of these advances has

been the survival rate of premature infants born before the 24th week of gestation who represent the highest risk group for pathogenesis of BPD. This means that the number of infants born with an immature lung (in the canalicular stage of lung development) is increased, leading to an increase in the incidence of BPD. Thus, it is urgent to find a treatment for BPD. The treatment of BPD has been confounded by its multifactorial causes. Therefore, only a comprehensive and individualized therapy adjusted to the profile of risk factors of each prematurely born infant will likely be able to provide a meaningful and effective strategy. The early identification of infants predisposed to BPD is essential. Several studies have been conducted to detect biomarkers in premature infants indicating their level of risk for BPD (132). Yet, the results remain inconclusive due to the low numbers of infants considered for the study. Importantly, more effort should be made in establishing preventive therapy. For example, more research should be conducted to understand the pathogenesis of preterm labor, a common cause of preterm delivery, which is one of the main risk factor associated with BPD. In addition, the current knowledge about the mechanisms of alveolarization must be expanded. Due to lack of human lung samples, the establishment of animal models precisely resembling the disease condition of BPD in humans will be helpful. Single cell transcriptomic studies should be carried out on alveolar myofibroblasts in physiological and pathological conditions to unravel the aberrant gene expression patterns and/or gene mutations responsible for impaired secondary septae formation. Furthermore, the use of the pneumonectomy mouse model and cell specific lineage-tracing approaches to understand the process of *de novo* alveolarization should contribute significantly to our understanding of lung regeneration. Last, but not least, the knowledge about progenitor/stem cells located in niches of the postnatal lung will be a valuable source of information that would be useful in triggering lung regeneration subsequent to injury.

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GENERAL COMMENTARY
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Commentary: A Breath of Fresh Air on the Mesenchyme: Impact of Impaired Mesenchymal Development on the Pathogenesis of Bronchopulmonary Dysplasia

*Eric S. White**

Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI, USA

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Anne Hilgendorff, Helmholtz Zentrum München, Germany

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***Correspondence:**
Eric S. White
docew@umich.edu

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A commentary on

A breath of fresh air on the mesenchyme: impact of impaired mesenchymal development on the pathogenesis of bronchopulmonary dysplasia
*by Chao C-M, El Agha E, Tiozzo C, Minoo P, Bellusci S. Front Med (2015) **2**:27. doi: 10.3389/fmed.2015.00027*

Bronchopulmonary dysplasia (BPD) is a chronic lung disease of prematurity that is only now beginning to be understood at the molecular level. The review by Chao et al. (1) elegantly takes us through mouse and human lung development to identify possible mediators of the abnormal mesenchymal response characteristic of the disease. However, it quickly becomes clear that many aspects of BPD development seem to mimic chronic lung disease in adults. For example, secondary septa formation is a critical step in alveologenesis, with alveolar simplification in BPD thought to result from disordered septation. In the emphysematous adult lung, enlarged airspaces reminiscent of alveolar simplification are also seen; importantly, evidence suggests that many mediators thought to be important in BPD development are also important in COPD pathogenesis, such as wingless and int (Wnt) (2, 3), fibroblast growth factor (FGF) (4, 5), and sonic hedgehog (Shh) (6). Similarly, exuberant collagen and elastin deposition during alveologenesis bears remarkable similarity to the deposition of extracellular matrix proteins during adult lung fibrogenesis (7), although it is unclear whether the pattern and relative quantities of proteins are also similar. These observations, plus others, suggest a potential stereotypic lung injury response resulting in disrepair in adult lung and abnormal development in the neonate.

Despite the differences in etiology, both BPD and chronic adult lung injury are characterized by elevated expression and activity of transforming growth factor (TGF)-β, perhaps the most well-known profibrotic cytokine. Implicated in epithelial–mesenchymal transition (EMT) (8), myofibroblast differentiation (9), and epithelial apoptosis (10), TGF-β overexpression may be pathogenic for BPD, emphasizing the critical need for TGF-β regulation during fetal lung development (11). Similarly, in adult lung, TGF-β overexpression is maladaptive, resulting in fibrosis that has been likened to recapitulation of developmental programs (12). Understanding how dysregulated TGF-β activity perturbs both normal development and postnatal lung repair has yet to be determined, but side-by-side comparisons of the role of mesenchymal cells in lung development and disease will likely shed further insight.

An intriguing aspect of the review by Chao et al. is the supportive role of the lipofibroblasts (LIFs), the interstitial fibroblasts identified in rodent lungs containing cytoplasmic lipid droplets (13) thought to be important in alveolar epithelial cell surfactant production. Despite one manuscript showing the existence of LIFs in human lung (14), controversy still remains (15) about the existence of this cell in humans. While LIFs seem to contribute to secondary septation in developing rodent lungs through transfer of lipids to Type II alveolar epithelial cells (16), their role in the adult rodent lung (if any) remains unclear. To be certain if LIFs are developmentally important and potentially reparative mesenchymal cells of the lung, identifying them in human lung of any age would be of paramount importance. Similarly, it will be important to determine whether LIFs are truly a separate type of mesenchymal cell or whether they are simply interstitial fibroblasts that uptake lipid droplets for transfer to alveolar epithelium. Currently, there are few known markers of LIFs, such as platelet-derived growth factor receptor (PDGFR)-α and peroxisome proliferator-activated receptor (PPAR)-γ (17), but these are not specific for LIFs. More work in this area will obviously be necessary.

Mesenchymal–epithelial crosstalk, as described above for LIFs and type II alveolar epithelial cells, is obviously important for lung development, as nicely illustrated by Chao and colleagues (1). However, less is known about the contribution of the endothelium, and more specifically mesenchymal–endothelial interactions, in the development of chronic lung diseases, including BPD. Clearly, the transformation of a double vasculature to a single capillary system in primary alveolar septae is integral to gas exchange in the developing lung, as pointed out by Chao and colleagues. The development of this vasculature occurs through both angiogenesis and vasculogenesis. Along the same line, angiogenesis and vasculogenesis appear to be important in the development of certain adult chronic lung diseases, such as COPD, asthma, and idiopathic pulmonary fibrosis (IPF) (18–20). Indeed, nintedanib, a tyrosine kinase inhibitor that blocks vascular endothelial growth factor receptor, FGF receptor, and PDGFR, has recently gained approval around the world for treatment of IPF because of its effects on slowing the rate of decline of lung function (21). It is not yet known whether the salutary effect of nintedanib in IPF is related to its inhibitory effects on angiogenesis.

We have certainly learned much about BPD pathogenesis through elegant mouse modeling and human pathologic studies. However, there is still much to learn; murine studies, while informative, cannot take the place of knowledge generated in human samples of disease. Moreover, although mouse lung development occurs in a stereotypic fashion which has been well characterized, it is not proven that human lung development occurs entirely in the same way. Thus, better methods of studying human lung development need to be created and validated, with findings hopefully informing our understanding of chronic adult lung diseases as well.

As alluded to by Chao and colleagues (1), the incidence of BPD and its long-term effects appears to be on the rise as more premature infants survive owing to advances in supportive care. There are no definitive therapies for these patients. Just as research into the lung mesenchyme will surely enlighten our understanding of BPD, researchers of all chronic lung diseases are likely to gain better insights into disease pathogenesis and potentially pave the way for future therapies in numerous diseases. Impaired mesenchymal development and responses – it is not just for BPD.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Updates on functional characterization of bronchopulmonary dysplasia – the contribution of lung function testing

Anne Greenough[1,2]* *and Anoop Pahuja*[3]

[1] Division of Asthma, Allergy and Lung Biology, MRC and Asthma UK Centre in Allergic Mechanisms of Asthma, King's College London, London, UK, [2] NIHR Biomedical Research Centre, Guy's and St. Thomas NHS Foundation Trust, London, UK, [3] Neonatal Intensive Care Centre, King's College Hospital NHS Foundation Trust, London, UK

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that predominantly affects prematurely born infants. Initially, BPD was described in infants who had suffered severe respiratory failure and required high pressure, mechanical ventilation with high concentrations of supplementary oxygen. Now, it also occurs in very prematurely born infants who initially had minimal or even no signs of lung disease. These differences impact the nature of the lung function abnormalities suffered by "BPD" infants, which are also influenced by the criteria used to diagnose BPD and the oxygen saturation level used to determine the supplementary oxygen requirement. Key also to interpreting lung function data in this population is whether appropriate lung function tests have been used and in an adequately sized population to make meaningful conclusions. It should also be emphasized that BPD is a poor predictor of long-term respiratory morbidity. Bearing in mind those caveats, studies have consistently demonstrated that infants who develop BPD have low compliance and functional residual capacities and raised resistances in the neonatal period. There is, however, no agreement with regard to which early lung function measurement predicts the development of BPD, likely reflecting different techniques were used in different populations in often underpowered studies. During infancy, lung function generally improves, but importantly airflow limitation persists and small airway function appears to decline. Improvements in lung function following administration of diuretics or bronchodilators have not translated into long-term improvements in respiratory outcomes. By contrast, early differences in lung function related to different ventilation modes have led to investigation and demonstration that prophylactic, neonatal high-frequency oscillation appears to protect small airway function.

Keywords: bronchopulmonary dysplasia, prematurity, resistance, compliance, diuretics, bronchodilators, corticosteroids

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***Correspondence:**
Anne Greenough, NICU, King's College Hospital, 4th Floor Golden Jubilee Wing, Denmark Hill, London SE5 9RS, UK
anne.greenough@kcl.ac.uk

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Introduction

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that predominantly affects prematurely born infants, but can occur in those born at term if they are subjected to high-inflation pressures. Initially, BPD was described in prematurely born infants who often had suffered severe respiratory

failure and required high pressure, mechanical ventilation with high concentrations of supplementary oxygen, often coined old BPD. Such infants were not routinely exposed to either antenatal corticosteroids or postnatal surfactant. BPD now also occurs in very prematurely born infants who initially had minimal or even no signs of lung disease, the so-called new BPD (1). At postmortem, infants with new rather than old BPD have less interstitial fibrosis, but an arrest in acinar development, resulting in fewer and larger alveoli (2). The chest radiograph (CXR) appearance of new BPD, that is small volume, hazy lung fields, is very different from the cystic abnormalities and interstitial fibrosis seen in "old" BPD. As a consequence, lung function abnormalities are likely to differ according to whether an infant is developing old or new BPD. The nature of the lung function abnormalities may also be influenced by the criteria used to diagnose BPD. These have included oxygen dependency at 28 days or 36 weeks post conceptional age (PCA) with or without radiological abnormalities. Nowadays, there is a consensus that infants should be diagnosed as having BPD if infants are oxygen dependent at 28 days after birth (3). They are then classified as suffering from mild, moderate, or severe BPD according to their respiratory support requirement at a later date (36 weeks PCA if born prematurely) (3) (**Table 1**). A further problem is that different levels of oxygen saturation have been used to determine the need for supplementary oxygen leading to wide variations in the occurrence of BPD (4). As a consequence, it is now recommended that an oxygen reduction test is used to determine whether supplementary oxygen is still required (5). Of note, lung function abnormalities as assessed by pulmonary function testing are not part of the current criteria to diagnose BPD, likely reflecting pulmonary function testing is not routinely available in all neonatal intensive care units (NICU).

Bearing in mind those caveats, an aim of this review is to describe lung function abnormalities in infants developing or with established BPD and how they change with increasing postnatal age during infancy. In addition, we will highlight if lung function testing in the NICU and during infancy gives added value. For example, do lung function test results so accurately predict BPD development that they could be used to identify infants who would benefit from intervention strategies or have improvements in lung function given an early indication of clinically efficacious interventions.

It, however, should be emphasized at the outset that a diagnosis of BPD is a poor predictor of ongoing pulmonary problems (6) and infants with and without BPD suffer respiratory morbidity at follow-up. In addition, certain randomized controlled trials (RCTs), which have demonstrated a reduction in BPD, have not been associated with improvements in long-term respiratory outcome (7) and equally interventions influencing long-term respiratory morbidity were not associated with a reduction in BPD (8, 9).

TABLE 1 | BPD severity modified from Jobe and Bancalari (3).

Infants <32 weeks of gestational age are assessed at 36 weeks PCA or at discharge home, whichever came first

Infants born at 32 weeks of gestation or greater are assessed at 56 days postnatal age or discharge home, whichever came first

The severity of BPD being graded in both groups accordingly

- mild BPD – breathing air
- moderate BPD – requirement for <30% supplementary oxygen
- severe BPD – requirement for more that 30% oxygen and/or positive pressure ventilation or nasal continuous positive airway pressure (CPAP)

Appropriate Lung Function Tests

Key to interpreting lung function data is whether an appropriate test has been used and whether the test has been applied robustly. In infants with evolving or established BPD, the techniques have strengths and weaknesses. Dynamic lung compliance measurements do not require airway occlusions, which may be poorly tolerated in infants with respiratory distress. Esophageal pressure measurements, however, are required, which may not accurately reflect pleural pressure changes in prematurely born infants who have a floppy chest wall or in the presence of lung disease. Single breath mechanics do require airway occlusions and the underlying assumptions are invalid if the respiratory system cannot be described as single-compartment model. The high resistance of small endotracheal tubes (10) may invalidate attempts to detect small changes in resistance in intubated infants. Assessments of functional residual capacity (FRC) by gas dilution or washout, can be applied in ventilated infants, but may underestimate the FRC if insufficient time is allowed for complete equilibration (11). Nitrogen washout with pure oxygen is impractical for ventilated infants receiving a high-fractional inspired oxygen concentration and inappropriate for infants at risk of retinopathy of prematurity. Inert gases such as helium and sulfur hexafluoride (SF6) avoid these problems. As a relatively heavy gas with low diffusivity, SF6 has the additional advantage of being less susceptible than helium to leaks (12), especially those occurring around an uncuffed endotracheal tube. Certain centers, however, use shouldered tubes, which have been demonstrated to have minimal or no leak (13). The major strength of measuring lung volume using infant whole-body plethysmography is that the total lung volume can be measured and hence, if used in conjunction with a gas dilution technique, can provide an assessment of hyperinflation and gas trapping (14). Systems are commercially available, but depend on electronic manipulation to close the pressure flow loop, which can result in erroneous results (15). A further disadvantage is that plethysmographs are not suitable for cot side measurements. In addition, the accuracy of plethysmographic measurements is dependent on rapid equilibration of pressures during respiratory efforts against occlusions, so that pressure changes at the airway opening reflect those in the alveoli (16). In the presence of severe airway obstruction, this may not occur, resulting in a phase lag between airway pressure and box volume, usually resulting in overestimation of lung volume (11). As infants actively elevate their end expiratory level, all lung volume measurements should be made during quiet, non-rapid eye movement sleep (17). Respiratory impedance plethysmography (RIP) can provide information on respiratory rate and the degree of thoracoabdominal asynchrony. The interpretation of the results is dependent on sleep state and volume calibration is not possible in this population.

Lung Function Abnormalities

Among infants with "old" BPD, increased resistance in the first week after birth and increased total respiratory and expiratory resistance with severe flow limitation, especially at low lung volumes at 28 days after birth was reported (18). More recent studies, which have included infants who usually have received surfactant, have demonstrated somewhat differing results. In a group of infants, the majority of whom had been given rescue surfactant, respiratory system resistance (Rrs) was abnormal at 10 days after birth, but then there was progressive improvement to normal values (19). In a series in which infants were given prophylactic surfactant and exposed to antenatal steroids, Rrs differed significantly between those who did and did not develop BPD on day three, but not at 14 days after birth (20).

Compliance is initially low in infants destined to develop BPD. In one series of ventilated infants, compliance of the respiratory system (Crs), using the single breath technique, was 50% of predicted at 10 days of age (19). Interestingly, there was a positive correlation ($r = 0.8$, $p < 0.001$) between those Crs results and maximal flow at FRC (V'_{max} FRC) using the forced expiratory volume technique at 2 years of age. In the presence of low-saccular compliance, the highly compliant distal bronchial tree is preferentially over distended resulting in marked distortion of both distal and central areas during mechanical ventilation (21). The authors (19), therefore, postulated that in infants with very low lung compliance in the neonatal period, cyclic bronchiolar stretching during positive pressure ventilation resulted in terminal airway ischemia and necrosis and subsequent fibrosis and smooth muscle hypertrophy. Compliance and lung volume abnormalities may persist over the neonatal period. Comparison of FRC results from 16 BPD infants (oxygen dependent for more than 28 days) and 8 infants without BPD demonstrated the BPD group had lower FRCs at both 14 and 28 days (22). Similarly, serial measurements of Crs and FRC in 74 infants, median gestational age 30 weeks, 35 of whom developed BPD (23 had moderate/severe BPD) demonstrated that those developing BPD, particularly moderate/severe BPD had significantly lower Crs and FRC results throughout the neonatal period compared to those who did not develop BPD (20). CXR thoracic areas and FRC measurements assessed in the first 72 h after birth in 53 infants, median gestational age of 28 weeks, also demonstrated lower FRCs in the BPD group, but the CXR thoracic areas were higher in the infants who subsequently developed BPD (oxygen dependency at 28 days) perhaps indicating gas trapping. The differences were particularly marked in infants who developed moderate/severe BPD (23). The reduced Crs in the neonatal period may be due to ongoing surfactant abnormalities, edema, and atelectasis. Similarly, the initial functional lung volume is likely reduced because of atelectasis.

During evolving BPD, there is gas trapping. FRC measured by plethysmography (FRCpleth) has been reported to be elevated in infants with BPD (24, 25) and in an early study FRCpleth was higher than FRC assessed by nitrogen washout (26). In established BPD, functional lung volume around term equivalent has been reported to still be significantly reduced compared, to data, from healthy term born infants both in an early (27) and in a more recent (24) study and associated with disturbed gas mixing (28). Those results appear pertinent to the present population of prematurely born infants who develop BPD. In a subsequent study (29), approximately 50% of the infants were exposed to antenatal steroids and 100% of those who developed moderate or severe BPD received surfactant. At term corrected, the severe BPD group had lower FRC, less efficient gas mixing, and higher specific conductance than those with mild and moderate BPD or the prematurely born controls. The infants with mild or moderate BPD infants also differed from the controls (29). BPD infants have also been shown to have significant increases in FRC, residual volume (RV), and RV/total lung capacity, which were more marked in those with recurrent wheeze, suggestive of hyperinflation and air-trapping (24). In a follow-up of prematurely born infants all born at <29 weeks of gestational age, two-thirds of whom had BPD, the degree of gas trapping significantly correlated with days of wheeze (30).

Results from a small study suggest that single photon emission computed tomography (SPECT) may provide additional information about regional lung function in BPD infants (31). SPECT was used to measure the distribution of lung ventilation (V) and perfusion (Q) in 30 BPD infants at a median PCA of 37 weeks. An unsatisfactory V/Q match was not correlated with the time spent on supplemental oxygen or CPAP, but was significantly negatively correlated with the time spent on mechanical ventilation. Increasing severity of BPD, however, was not consistently associated with the degree of V/Q mismatch.

Other lung function abnormalities in infancy suggest impairment of alveolar development after very premature birth. Pulmonary diffusing capacity and alveolar volume were assessed at 11.6 months of age using a single breath hold maneuver at elevated lung volume in 39 BPD infants (oxygen requirement at 36 weeks) and 61 term born controls. The BPD patients had reduced pulmonary diffusing capacity when adjusted for body length or alveolar volume (32).

Longitudinal Assessment

Early studies assessing serial lung function highlighted that lung compliance and FRC improved with increasing age (19, 27), such that by 2 years of age they had reached the normal range (19). In addition, during the first 2 years after birth, a relative increase in FRC using a gas dilution technique was reported (19). More recently, results were apparently at variance as assessment of 55 sedated VLBW infants (29 with BPD, oxygen dependency at 36 weeks PCA) at 50, 70, and 100 weeks of PCA demonstrated significantly lower tidal volume, minute ventilation, compliance, and FRC results in the BPD infants (33). Those differences, however, were no longer statistically significant once the results were normalized for body weight, which was significantly lower in the BPD group (33).

Airflow limitation, however, appears to be a persisting problem. An early report highlighted that lower airway obstruction persisted in infants with BPD who had severe disease as indicated by requirement for a tracheostomy (34). Longitudinal assessment demonstrated that, in infants with severe BPD, abnormalities in forced vital capacity (FVC) took longer to improve them eventually reaching the normal range by 3 years of age, but there was no

improvement in forced expiratory flow at 75% of vital capacity (FEF_{75}) over the study period (34). In another study, 70% of BPD infants assessed at 2 years had low flow rates below 40% of that predicted, whereas lung volumes, Crs, and Rrs results were in the normal range (19). More recent results in the present population of prematurely born infants confirm those results (25, 34). Assessment of 44 children with BPD (oxygen dependency at 28 days or 36 weeks PCA with CXR changes) highlighted that there were no improvements in V'_{max} FRC at 6, 12, and 24 months (35). In a longitudinal study, which examined infants at a PCA of 58 weeks and then 33 weeks later, the group mean lung volumes and flows tracked at or near their previous values, that is, there was a lack of catch up growth. There were, however, improvements in lung function in those with above average growth (25).

Serial lung function measurements in infants with BPD have shown a decline in small airway function (as evidenced by assessments of V'_{max} FRC) during the first year after birth (36). Similar changes in small airway function, however, have been reported in healthy, unsedated, prematurely born infants (37). Those findings emphasize the importance of using an appropriate control group when interpreting long-term effects of respiratory disease or management strategies in the neonatal period.

Prediction of BPD

Initial studies focused on compliance and resistance results with differing results. In certain studies, resistance, but not compliance, results were predictive of BPD development. One study, however, included only 20 infants who had required mechanical ventilation for at least 3 days; 8 developed BPD (supplementary oxygen for longer than 28 days) (38). In a study of 46 infants with a birth weight <1.0 kg, those who subsequently developed BPD had significantly higher Rrs, but not Crs, at 1 week and Rrs was significantly higher in those with evolving BPD throughout the neonatal period (39). In another study, Rrs but not Crs before surfactant therapy was associated with an increased risk of BPD. Areas under receiver operator characteristic (ROC) curves were reported and demonstrated that Rrs performed similarly to gestational age and birth weight (40).

By contrast, other studies have demonstrated compliance rather than resistance was predictive of BPD development. Dynamic compliance, assessed using flow measurements from a pneumotachograph related to mean airway pressures in 47 infants mean gestational age of 30 weeks in the first 3 days after birth, was significantly lower in those who developed BPD, which was diagnosed using radiological criteria (41). In a subsequent study, an occlusion technique was used to determine appropriate esophageal balloon placement and longitudinal assessment over the first month was made on 143 infants with a gestational age of 27–30 weeks. The model, which included gestational age and dynamic pulmonary compliance, had the highest positive predictive accuracy (100%) for BPD (the need for supplemental oxygen at 4 weeks of age), whereas the predictive value of total pulmonary resistance was minimal (42). In 39 ventilated infants with a mean gestational age 26–28 weeks, the predictive ability of the results of the interrupter technique was compared to respiratory mechanics results obtained during mechanical ventilation. Dynamic compliance of the respiratory system on day 1, birth weight and gestational age were all significantly lower in the BPD infants (BPD diagnosed if the infant developed lung disease in the first week after birth, was oxygen dependent at 28 days of age and developed characteristic chest x-ray changes), but there were no significant differences in the interrupter technique results. Dynamic compliance of the respiratory system was a better independent predictor of BPD development than gestational age or birth weight (43). More recently, among 52 prematurely born infants who were ventilated for more than 72 h and had received a single dose of porcine surfactant, initial compliance results did not differ between infants who went on to develop mild or severe BPD, but at days 7 and 10 the "severe" group had significantly poorer compliance results. BPD was diagnosed as a requirement of supplementary oxygen at 28 days to maintain oxygen saturation above 95%; the severity of BPD was determined by the CXR score. Compliance but not resistance on days 7 and 10 were predictive of severe BPD (44).

In other studies, neither compliance nor resistance results were predictive of BPD development (45, 46). In a study of 104 ventilator-dependent infants, with a mean gestational age 27–30 weeks, respiratory mechanics were measured using an airway occlusion technique between 6 and 48 h after birth and corrected for body length. Birth weight, but not respiratory system mechanics, predicted BPD development (supplemental oxygen requirement 28 days after birth) (45). In a series of 58 infants who had RDS, compliance and resistance results were assessed using a commercially available system. BPD was diagnosed as oxygen supplementation, respiratory distress, and an abnormal CXR at 28 days. Neither lung compliance nor pulmonary resistance on days 1–4 predicted BPD, but gestational age and a ventilatory index on day 3 (ventilator frequency × maximum inspiratory pressure) were predictive (46).

It is not possible to conclude from the above studies whether assessment of lung mechanics is helpful in predicting BPD development. The discrepancy in the results likely reflects that different techniques were used in different populations. Few of the above studies included a sample size calculation and thus there may have been both type I and II errors.

More recent studies have investigated whether assessment of FRC might be predictive of BPD. In 100 infants with a median gestational age 28 weeks and ventilated within 6 h of birth, FRCHe ≥19 ml/kg and a low gestational age in the first 48 h were more accurate predictors of BPD at 28 days than Crs or Rrs. Indeed, if only the 50 infants whose gestational age was ≤28 weeks of gestation were considered, a low FRC on day 2 was the best predictor of BPD development (47). Subsequently, the results of FRC and Crs on days 3 and 14 after birth were compared to a marker of inflammation, end-tidal carbon monoxide (ETCO). Seventy-eight infants with a median gestational age of 29 weeks were assessed; 39 developed BPD (oxygen dependency at 28 days); a sample size calculation was given. Gestational age, birth weight, ETCO, FRC, and Crs results on days 3 and 14 differed significantly between those who did and did not develop BPD. Multifactorial logistic analysis, however, demonstrated only birth weight and ETCO levels on day 14 were significant predictors of BPD with an area under the ROC curve of 0.97. Those ETCO results indicate ongoing inflammation in infants developing BPD (48).

Response to Therapies

Lung function tests have been used to assess the response to therapies in infants with evolving or established BPD. Variable and often conflicting results have been reported, which reflects the use of different techniques, some of which were inappropriate in this age group, lack of a sample size, or an inappropriate sample size based on too optimistic a view of the likely effect. A further problem in interpreting the results are that many of the studies were reported more than 20 years ago and, therefore, include infants not exposed to antenatal steroids or postnatal surfactant, which could have affected their response to the intervention. More importantly, reported changes in lung function results were not always accompanied by a change in clinical status or affected longer-term outcome.

Diuretics

Bronchopulmonary dysplasia infants often are poorly tolerant of fluid loads with excessive weight gain on standard fluid regimens. As a consequence, diuretics are frequently prescribed but have short- and long-term side-effects including electrolyte disturbance and nephrocalcinosis. It is, therefore, important to determine if they are having a positive effect. Early results demonstrated that administration of frusemide acutely increased lung compliance and reduced airway resistance (49, 50) was associated with a reduction in ventilator requirements (51) and transient improvements in blood gases (49). A systematic review, however, demonstrated that in prematurely born infants <3 weeks of age, frusemide administration had either inconsistent or no detectable effects (52). In addition, in 16 spontaneously breathing infants with postnatal ages ranging from 4 to 35 weeks, a single dose of frusemide was associated with improvement in pulmonary compliance but not blood gases or resistance. Furthermore, a 6- to 10-day course was associated with improvement in compliance and resistance (53), but better oxygenation was only achieved in 6 of the 16 infants.

Nebulized frusemide has been given with the hope that this would improve respiratory function while avoiding the systemic complications. In a study of eight ventilated infants with a mean postnatal age of 33 days, the effects of 0.1, 0.25, 0.5, and 1.0 mg/kg of nebulized frusemide were assessed. A dose of 1 mg/kg was associated with a 28% improvement in pulmonary resistance and a 51% improvement in pulmonary compliance at 1 h; the effects lasted for least 4 h. A systematic review demonstrated a significant improvement in tidal volume after 1 and 2 h, but no improvement in compliance at either time point (54).

Due to the side-effects of frusemide, infants who require chronic diuretic therapy are often changed to chlorothiazide and spironolactone. It had been suggested that the combination improved the outcome of babies with severe BPD (55). In a randomized, double blind, crossover trial, the effects of oral diuretics (chlorothiazide 20 mg/kg/dose and spironolactone 1.5 mg/kg/dose) given twice daily for a week were compared to placebo (56). The mean airway resistance, specific airway conductance, and dynamic compliance improved significantly, but only 10 infants were included in the study. In a further randomized trial (57), spironolactone and chlorothiazide were compared to placebo in 43 oxygen dependent BPD infants. Infants in the treatment group only had improvements in dynamic pulmonary compliance and airway resistance and, at 4 weeks after study entry, required less supplementary oxygen than the placebo group. There were, however, no significant differences in the pulmonary function test results after discontinuation of treatment, nor in the total number of days supplementary oxygen was required between the two groups. Orally administered diuretics (chlorothiazide and spironolactone) in combination with theophylline have been demonstrated to have an additive positive effect on dynamic compliance, but no effect on clinical outcomes were reported (58).

Bronchodilators

Inhaled bronchodilators have been reported to improve pulmonary resistance, dynamic compliance, and transcutaneous blood gases when administered to ventilated babies with BPD at approximately 1 month of age (59) and reduce airway resistance in infants with BPD at term (60–62). Intravenously administered salbutamol (30 μg/kg) in six infants aged between 54 and 105 days resulted in an improvement in respiratory system compliance and resistance using the occlusion technique, but there was no correlation between salbutamol serum concentration and pulmonary function changes (63). Comparison of the effectiveness of aerosol and intravenous delivery of salbutamol was made in eight ventilator-dependent infants in randomized order; there were similar improvements in pulmonary mechanics with the two delivery methods (64). In a comparison of different inhalation devices in infants during unassisted breathing as well as in a group of ventilated infants (65, 66), it was reported that both Crs and Rrs were sensitive indicators of a bronchodilator effect. Interpretation of those data is, however, limited because the studies were performed prior to standardization of the technique and intra-individual variability was not reported.

Synergism was reported between ipratropium bromide (IB) and salbutamol in improving pulmonary mechanics in ventilated infants for up to 1–2 h after administration (67). In 10, ventilator-dependent infants, mean age 25 days, various dose of IB (75, 125, and 175 μg) plus 0.04 mg salbutamol were compared to placebo. Rrs and Crs were measured by the single breath occlusion technique. The greatest decline in Rrs (mean 26%) was seen after 175 μg IB with salbutamol. The authors, therefore, concluded that muscarinic receptors contribute to the increased bronchomotor tone of infants with BPD (67). No synergy, however, was shown between metaproterenol and atropine with lung function returning to baseline after both treatments (68).

All of the above studies were undertaken more than 20 years ago, there were no sample size calculations and no long-term benefits reported. As a consequence, in the present population of premature infants, diuretics should only be given to treat incipient heart failure in infants with evolving or established BPD and stopped as soon as that problem ceases. Equally, bronchodilators should only be administered to treat troublesome wheeze and continued if the administration is associated with a reduction in respiratory support requirements. Administration should be via a metered dose inhaler and spacer rather than a nebulizer, as the nebulizing fluid can cause bronchoconstriction (69).

Dexamethasone

The efficacy of dexamethasone to prevent and treat BPD has been tested in many RCTs. Unfortunately, although systemically administered steroids have many positive effects, they have short- and long-term adverse effects. As a consequence, attention has been given to assessing the response to lower doses and inhaled steroids. A 1-week tapering course of dexamethasone starting at 0.5 mg/kg/day given at 7–14 days of age in ventilator dependent, VLBW infants increased pulmonary compliance and decreased the incidence of BPD at 36 weeks PCA (70). In a subsequent study, the effectiveness of that dose (total dose 2.35 mg/kg) compared to a lower dose (total dose 1 mg/kg) was compared. FRC using a nitrogen washout technique and Crs by an occlusion technique were measured in infants at a mean age of 11 days. The sample size was powered to detect that the increase in FRC in the lower dose group would be more than 10% smaller than in the higher dose group. No significant differences were shown (71). In a RCT, 10 days of dexamethasone were compared to 100 μg qds per day of budesonide in 40 infants with a median postnatal age 27 days. The study was powered to detect a difference of 7% in the inspired oxygen requirement 1 week after starting therapy. After 36 h, only the systemic group had significant reductions in the inspired oxygen concentration and Crs and at 1 week the systemic group had significantly better results than the inhaled group (72).

Respiratory Support

Lung function testing has been used to compare the acute and longer-term efficacy of respiratory support techniques. For example, in a randomized crossover study, proportional assist ventilation (PAV) and assist control ventilation (ACV) were examined in infants with evolving BPD. When on PAV the infants had superior respiratory muscle strength and a lower work of breathing and this was associated with better oxygenation (73). Follow-up studies assessing the efficacy of ventilation modes demonstrate the importance of which lung function technique was employed. In a follow-up study of an RCT, no advantage of HFO over conventional ventilation (CMV) was reported with respect to lung function at 1 year corrected, but small airway function was only assessed by evaluation of gas trapping (74). In another study, V'_{max} FRC was assessed at 6 and 12 months in infants who developed BPD. Those who were initially supported by CMV showed the expected decline in small airway function, but this was not seen in those seen in the HRO group. As a consequence, at 12 months, the HFO group had superior lung function (75). The results of that non-randomized study suggested that HFO might protect small airway function. Follow-up at 11–14 years of children who had been entered into a neonatal RCT of prophylactic HFO has subsequently proved that hypothesis (9).

Conclusion and Future Directions

- Infants who develop BPD have low compliance and lung volumes and elevated resistances in the neonatal period.
- During infancy, lung function generally improves, but airflow limitation persists and small airway function declines.
- Improvements in lung function following administration of diuretics or bronchodilators have not translated into long-term improvements in respiratory outcomes, but assessment at follow-up has demonstrated neonatal high-frequency oscillation appears to protect small airway function.
- Further investigation should be undertaken to determine whether a lung function assessment accurately predicts chronic respiratory morbidity and hence prophylactic interventions can be appropriately targeted.
- Lung function assessment at follow-up should be incorporated into all neonatal RCTs, which are aimed at improving respiratory outcome.

Author Contributions

Both authors undertook a literature review and produced the manuscript.

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Stem cells and their mediators – next generation therapy for bronchopulmonary dysplasia

Marius A. Möbius[1,2,3] *and Bernard Thébaud*[3,4]*

[1] Department of Neonatology and Pediatric Critical Care Medicine, Medical Faculty, University Hospital Carl Gustav Carus, Technische Universität Dresden, Dresden, Germany, [2] DFG Research Center and Cluster of Excellence for Regenerative Therapies (CRTD), Technische Universität Dresden, Dresden, Germany, [3] Regenerative Medicine Program, Sprott Centre for Stem Cell Research, Ottawa Hospital Research Institute, University of Ottawa, Ottawa, ON, Canada, [4] Division of Neonatology, Department of Pediatrics, Children's Hospital of Eastern Ontario, University of Ottawa, Ottawa, ON, Canada

Bronchopulmonary dysplasia (BPD) remains a major complication of premature birth. Despite great achievements in perinatal medicine over the past decades, there is no treatment for BPD. Recent insights into the biology of stem/progenitor cells have ignited the hope of regenerating damaged organs. Animal experiments revealed promising lung protection/regeneration with stem/progenitor cells in experimental models of BPD and led to first clinical studies in infants. However, these therapies are still experimental and knowledge on the exact mechanisms of action of these cells is limited. Furthermore, heterogeneity of the therapeutic cell populations and missing potency assays currently limit our ability to predict a cell product's efficacy. Here, we review the therapeutic potential of mesenchymal stromal, endothelial progenitor, and amniotic epithelial cells for BPD. Current knowledge on the mechanisms behind the beneficial effects of stem cells is briefly summarized. Finally, we discuss the obstacles constraining their transition from bench-to-bedside and present potential approaches to overcome them.

Keywords: bronchopulmonary dysplasia, lung, stem cells, mesenchymal stromal cells, endothelial progenitor cells, current good manufacturing practice, potency assay

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***Correspondence:**
Bernard Thébaud, Sprott Centre for Stem Cell Research, Ottawa Hospital Research Institute, CCW 6120, 501 Smyth Road, Ottawa, ON K1H 8L6, Canada
bthebaud@ohri.ca

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Introduction

The proper ventilation and oxygenation of a premature newborn is the foremost task in neonatology. But from the first breath of a premature newborn in the delivery room to the spontaneous or mechanical ventilation on the Neonatal Intensive Care Unit, the immature lung is always exposed to a non-physiological substance; it is not prepared for at this age: air, containing at least five times the oxygen concentration of the amniotic fluid (1). The abrupt confrontation of the immature lung to

Abbreviations: AEC, amnion epithelial cell; ARDS, acute respiratory distress syndrome; ATP, adenosine triphosphate; BMDAC, bone marrow-derived angiogenic cell; BOEC, blood outgrowth endothelial cell; BOS, bronchiolitis obliterans syndrome; BPD, bronchopulmonary dysplasia; CD, cluster of differentiation; CDH, congenital diaphragmatic hernia; CdM, conditioned media; cGMP, current good manufacturing practice; COPD, chronic obstructive pulmonary disease; CPC, circulating progenitor cell; ECFC, endothelial colony forming cell; EPC, endothelial progenitor cell; EpCam, epithelial cell adhesion molecule; FBS, fetal bovine serum, FiO_2 fraction of inspired oxygen; GvHD, graft-versus-host disease; IPF, idiopathic pulmonary fibrosis; MSC, mesenchymal stromal cell; PCR, polymerase chain reaction; PDGFR, platelet-derived growth factor receptor; SCID, severe combined immunodeficiency; SSEA, stage-specific embryonic antigen; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

this and other hostile extrauterine conditions leads to the chronic lung disease of prematurity or bronchopulmonary dysplasia (BPD).

Despite advances in the management of premature infants, respiratory complications still account for approximately one-quarter of all NICU deaths (2). BPD, characterized by impaired lung growth, remains the most common complication of premature birth (3, 4). Currently, there is no effective treatment for BPD and all present approaches remain either supportive, present major adverse effects (steriods) or show only small benefits (vitamin A, caffeine).

Cell-based therapies may open a completely new chapter in the therapy of BPD. Over the past years, animal studies using stem and progenitor cells as therapeutics showed very promising results, which have lead to first trials in human (5). This review summarizes our current knowledge about the therapeutic potential of these genuine facilitators of lung growth and regeneration.

Stem Cells – Origin of Growth, Repair, and Disease

Stem or progenitor cells reside in virtually all tissues at all stages of development. They are generally defined by the ability to (**I**) undergo self-renewal and (**II**) give rise to more differentiated cells. The extent to which these cells can differentiate is called potency. Stem cells harbor the potential to differentiate into placental and embryonic tissue (*totipotent* stem cells of the morula stage) and along the various embryonic germ layers (*pluripotent*, embryonic stem cells). They further give raise to several adult cell types (*multipotent*, i.e., hematopoietic stem cells). Conversely, progenitor cells are thought to give raise to only one specific adult cell type (*unipotent*, i.e., type 2 alveolar epithelial cells).

Toti-, pluri-, and multipotent stem cells enable early development of the embryonal structures and subsequent organ differentiation until the beginning of the fetal period approximately 8 weeks *post conceptionem*. After this period, derivates of these cells can be found as resident stem or progenitor cells in virtually all fetal and adult tissues deriving from all three germ layers, including the bone marrow (6), gut (7), brain (8), and lung (9).

Their major task is the facilitation of growth and of tissue regeneration and maintenance, e.g., providing new, differentiated cells after cell loss due to normal usage or injury while remaining in a proliferative, lesser differentiated state on their own (self-renewal). This happens to various extends. Some tissues – such as the gut or bone marrow – contain stem cells with high proliferative and regenerative capacity, while others – such as the brain and the heart – grow until adulthood, but have only limited regenerative potential once damaged.

The lung is a complex organ deriving from endodermal and mesodermal origin and harbors several endodermal (epithelial) and mesodermal (mesenchymal and endothelial) stem and progenitor cell types (10), each of them with different capabilities to differentiate and proliferate. As of now, over 40 different lung cell types have been described; numerous of them exert more or less characteristics of stem cells (9–11).

Since enabling growth and regeneration is the main role of stem or progenitor cells in non-embryonic tissues, organ failure would suggest a pathology of the organ resident stem cell population(s). Indeed, several events before (prolonged rupture of the membranes, oligohydramnios, severe intrauterine growth restriction, congenital diaphragmatic hernia/CDH) or after birth (mechanical ventilation, oxygen) may impair stem cell function. Lung diseases with abnormal growth of lung compartments, such as the bronchiolitis obliterans syndrome (BOS) following lung transplantation (12) or lung hypoplasia following CDH (13), can be linked to dysfunction of the resident progenitor cells.

In BPD, qualitative or quantitative impairment of resident mesenchymal and endothelial stem or progenitor cells seems to contribute to the disease pathogenesis or to the incapacity of the lung to repair itself (11). Less is known about the pathogenic role of stem or progenitor cells in the endodermal, epithelial lung compartments, such as the bronchioalveolar stem cells (BASCs) (14).

Conversely, exogenous stem cells or their products derived from the mesenchymal (14–26), epithelial (27–29), or endothelial (30, 31) compartment of easily accessible tissue, such as the bone marrow, placenta, or the umbilical cord prevent or restore lung damage in animal models of BPD. Most of these data have been generated in neonatal rodents exposed to hyperoxia, a model which will be discussed below. Newer models combining several factors contributing to BPD [such as antenatal hypoxia, inflammation, and mechanical ventilation (32, 33)] will be useful to assess the pathophysiology of BPD and therapeutic benefit of cell therapies more completely. Various cell therapies have been proposed (34), and the following paragraphs will focus on the most extensively explored therapeutic stem cells for BPD: mesenchymal stromal cells (MSCs), endothelial progenitor cells (EPCs, including endothelial colony forming cells, ECFCs), as well as amnion epithelial cells (AECs).

MSCs as Therapeutic Cells

Mesenchymal stem or stromal cells (MSCs) are the most promising cells in regenerative medicine. Their therapeutic potential is currently investigated in virtually every disease one can think of. As of February 2015, PubMed lists over 37,500 references for these cells; almost double the number from 2012 (35).

First described in hematopoietic tissues by Friedenstein and his colleagues in 1970 (36), MSCs have been identified in adult organs deriving from the mesodermal germ layer, including the bone marrow and adipose tissue. Furthermore, they can be found in fetal-restricted mesodermal derivates like the umbilical cord stroma and cord blood as well as in the placenta and the amniotic fluid [comprehensively reviewed by Hass and colleagues (37)]. Interestingly, MSCs have also been identified in tissues deriving from the (ectodermal) neural crest, such as the mandibula (38).

Cord-derived MSCs from the Wharton's Jelly are of particular interest for the treatment of neonatal diseases. Indeed, the umbilical cord stroma is

- readily available at birth and thus clinically relevant
- with 100 million births worldwide a large source of stem cells

- safe and painless to the mother and her child as cells are harvested after delivery from otherwise discarded tissue and thus devoid of ethical dilemma
- importantly, these cells hold superior healing capabilities compared to adult bone marrow cells (39).

As implied by their multiple residence tissues, MSCs represent a very heterogeneous cell population (40, 41). MSCs from one source exert different properties than MSCs from another (37, 42). Some cells within the MSC population are true stem cells with the potential to undergo complete self-renewal and some are not. Therefore, the global population of MSCs should be identified as "mesenchymal stromal cells" rather than "mesenchymal stem cells" (35).

The minimal criteria to define a MSC (41) are widely accepted, but relatively loose and include the following four:

- The ability of the cell to adhere and grow on plain, uncoated, tissue culture treated plastic surfaces, e.g., the ability to secrete large amounts of extracellular matrix.
- The presence of CD73, CD90 (thymocyte antigen *thy-1*), and CD105 (*endoglin 1*) on the cell's surface.
- The absence of the surface markers CD34, CD45, CD14/CD11b, CD19/CD79α, and HLA-DR, which label various cell lines from the hematopoetic lineage.
- The ability of the cells to differentiate along adipogenic, osteogenic, and chondrogenic lineages when stimulated *in vitro*.

These criteria were initially created to define MSCs derived from the bone marrow, where they need to be distinguished from the hematopoietic stem and progenitor cells giving rise to the blood cell lines. But as mentioned above, MSCs can also be found in other organs and tissues where they need to be distinguished from resident, mature fibroblasts, endothelial cells, and other non-hematopoetic cell types. Therefore, additional criteria for defining potentially therapeutic MSCs from, i.e., the umbilical cord or the adipose tissue, are required and currently under development. Several additional surface markers including CD10, CD29, CD106, CD146, CD166 or CD200 (42), and CD271 (43) have been proposed.

Bone marrow-derived MSCs exert a robust differentiation potential along osteogenic, chondrogenic, and adipogenic lineages. Conversely, some MSC populations can be differentiated into epithelial (44, 45), endothelial (46), and neural cells (47) while lacking the ability to differentiate along certain other, i.e., chondrogenic lineages (35). Therefore, criteria for a characterization by trilineage differentiation may need to be revised as well.

Functional tests, such as the assessment of the cell's immune-regulatory properties (48) and their secretome (49) following specific stimuli, gain importance and will open a new avenue for a functional, rather than a morphological description of potentially therapeutic MSC products. Nevertheless, a single, striking marker or feature to define an MSC has not yet been found; neither is there a valid test to assess the "stemness" or "therapeutic potential" of such a cell, a major problem, which will be discussed below.

Lung-Resident MSCs and the Development of BPD

Our current understanding of normal alveolar growth and the cellular and extracellular mechanisms behind its regulation suggest a crucial role of tissue-resident lung stem cells from mesenchymal, endothelial, and epithelial origin in this complex process (50, 51). Therefore, damage to the resident lung stem or progenitor cells – by inflammation, hyperoxia, malnutrition, shear stress, or other influences – may results in a loss or severe impairment of endogenous growth and regeneration potential.

The lung-resident MSC may play a critical role as regulator of lung development, coordinating epithelial and endothelial growth (52). When these cells become damaged in preterm infants, lung development gets out of sync leading to BPD. The properties of human neonatal and fetal lung MSCs are currently under investigation. While resident lung MSCs are by far not as well described as, i.e., BM-MSCs or adipose tissue-derived MSCs, pioneering work by Dr. Hershenson's group found that the presence of MSCs in the tracheal aspirates of ventilated preterm infants predicted BPD (53–55).

These cells express less platelet-derived growth factor-receptor alpha (PDGFR-α) as compared to MSCs from babies without BPD (56). Furthermore, they present a profound autocrine production of transforming growth factor beta 1 (TGF-1) (57) and increased β-catenin signaling (58). The disruption of these pathways controlling the myofibroblastic differentiation (PDGFR-α, TGF-β1, and β-catenin) leads to disrupted formation of alveolar tips and interstitial lung fibrosis (58, 59). Moreover, the function of specific lung-resident stem cells with mesenchymal, endothelial, and epithelial differentiation potential (lung side population cells) (60) is disrupted in murine hyperoxia-induced lung injury (61).

Therefore, these findings suggest that damage to endogenous MSCs may contribute to the disease pathogenesis of BPD. Conversely, exogenous MSCs show consistent therapeutic benefits in experimental neonatal lung injury models. How these exogenous MSCs affect resident lung MSCs is unknown.

Therapeutic Benefits of Exogenous MSCs

The beneficial effects of exogenous MSCs have best been described in hyperoxia-induced rodent models mimicking BPD (33, 62, 63). Rodents are convenient because they are born at the saccular stage of lung development, which corresponds to the lung developmental stage of a human infant born at 26–28 weeks of gestation (62). To summarize the models in brief, term born rodents are exposed to hyperoxia (FiO_2 0.60–0.95) for 1–2 weeks; rats or mice subsequently develop structural lung changes consistent with pathological findings of human infants that died with BPD (64). Alveolar simplification, capillary rarefaction, and leakage with extravascular fibrin and plasma protein accumulation, lung fibrosis with increased collagen and disordered elastin deposition, pulmonary hypertension, as well as influx of inflammatory cells can be observed (33, 62, 63).

A second model using prematurely delivered baboons model at 125 days and mechanically ventilated for 2 weeks offers unique opportunities to test promising (stem cell-based) therapies in a model close to the clinical setting (65). Due to the close relationship to man, long-term effects of treatment on growth and

development can easily be observed, giving valuable information for clinical applications in premature human infants.

Mesenchymal stromal cells have striking beneficial effects in the hyperoxia-induced model of BPD. In 2007, Tian et al. (26) reported that intravenous injection of bone marrow-derived MSCs ameliorates the oxygen-induced neonatal lung injury. Two papers published simultaneously by Aslam et al. (24) and van Haaften et al. (25) in 2009 demonstrated that MSCs derived from the bone marrow of healthy, adult rodents prevent oxygen-induced neonatal lung injury.

Both authors administered MSCs on postnatal day 4 before exposing the pups to hyperoxia to assess the preventive potential of the cells. Aslam and colleagues administered 5×10^4 cells (approximately 5×10^6 cells/kg bodyweight) intravenously, whereas van Haaften et al. used an intratracheal administration route and applied double the dose (1×10^7 MSCs/kg bodyweight). A significant decrease in alveolar wall thickness as well as an increase in vessel density and alveolar septation was observed in both studies. Furthermore, increased exercise capacity and reduced pulmonary hypertension was noted (18, 25).

Remarkably, very few of the injected cells were retained in the lung, indicating that cell engraftment contributes minimally – if at all – to the therapeutic benefit of MSCs. The intratracheal, intraperitoneal, or intravenous administration of cell free conditioned medium (CdM; concentrated tissue culture supernatant of MSCs) showed beneficial effects comparable to whole cell therapy. However, as no reliable methods to describe and normalize doses and composition of CdM have been utilized, a direct comparison of the two therapy regimens is inaccurate.

These experiments have been repeated several times with MSCs from the rat or human bone marrow (14, 17, 18, 21) or human cord blood (16, 22, 23, 66) and their respective conditioned media with similar results [reviewed by Fung et al. (67)]. Furthermore, recent pre-clinical studies by Chang and colleagues investigated the influences of the dose (22), timing (66), and administration route (23) of MSCs in a rat model of BPD. These studies favor an early, intratracheal administration of $0.5–5 \times 10^7$ MSCs/kg bodyweight.

MSCs and their CdM were also able to rescue hyperoxia-induced lung injury (16). Moreover, the beneficial effects of a treatment with these cells are not transient. Adult rats that received MSCs in their neonatal period before (16), during (17), or after exposure (16) to hyperoxia show persistent improvements in lung architecture, exercise capacity, and vascularization in long-term follow-up studies up to 6 months.

The exact mechanism behind the effects remains unclear. Secreted anti-inflammatory proteins, angiokines, and other lung protective substances including stanniocalcin-1 (19, 68), prostaglandin E2 (12), and TNF-stimulated gene/protein 6 (TSG-6) (69–71) are strongly suggested to account for the short-term effects and protect the lungs against the acute injury. These substances secreted by the MSCs blunt the immediate and oblique injury effects like the influx of inflammatory cells and their associated deleterious effects. This has not only been described in neonatal hyperoxic models but also in several other experimental studies using bleomycin (72), lipopolysaccaride (73), ovalbumin (74), or prolonged ventilation (75) to challenge the lung.

The pathophysiology of BPD is not limited to inflammation, despite a major contribution of this process to the development of the disease (76). BPD is a multi-factorial disease and the characteristic and life-impairing feature of BPD – compromised alveolar growth beyond the neonatal period – can best be explained by a persistent impairment of the mechanisms regulating lung growth and development, including the resident stem/progenitor cells.

The M&M's of Therapeutic Cells – Microvesicles and Mitochondria in Long-Term Effects of MSCs

As described above, very few cells engraft in the lung (25). The engrafted cells die rapidly and are not detectable with quantitative PCR methods or high-specific stainings after a few weeks when xenogeneic MSCs (=cells from a different species) were used (16). Authors using an allogeneic approach described a comparably low, but prolonged engraftment (up to 100 days) into the alveolar wall with potential transdifferentiation into surfactant-protein C producing cells (17, 25).

However, these events are very rare and do not contribute to the therapeutic effect of MSCs *in vivo* [reviewed by Kotton and Fine (77)]. Engraftment and transdifferentiation of MSCs may be considered as artifacts of the immunohistochemical detection method (78).

Microvesicles as Carriers of Therapeutic Agents

As discussed previously, secreted proteins mainly account for the short-term effects of transplanted MSCs or their CdM. But a long-term effect on the lung cells cannot be explained by just a single administration or secretion of cytokines. Extracellular vesicles, small microparticles containing nucleic acids, proteins, and lipids (79) may answer this question. Specific subtypes of these particles – so-called exosomes – are secreted by numerous cell types, including MSCs (80). They harbor the potential to reduce inflammation and blunt hypoxia-induced pulmonary hypertension (80) as well as to ameliorate endotoxin-induced lung injury (81).

Exosomes are, besides cytokines and other secreted proteins, the potential therapeutic components of conditioned medium. As reviewed comprehensively by Colombo et al. (79), exosomes can be taken up into the target cell by various mechanisms. Specific nucleic acids – so-called microRNA (82) – can transpose to the nucleus and silence specific genes for long periods (83) or interfere with the protein translation. These mechanisms could account for long-term beneficial effects on damaged lung cells in BPD.

Therapeutic Mitochondrial Transfer in Lung Disease

Another mechanism contributing to the long-term efficacy of MSCs may be the transfer of mitochondria from MSCs to damaged lung cells. Mitochondrial dysfunction plays a critical role in the development of experimental BPD in primates (84) and rodents (85–88).

In 2006, mitochondrial transfer from MSCs to other cells *in vitro* was described (89). Recent *in vivo* studies revealed that mitochondrial transfer plays a crucial role in animal models of lung injury. Intratracheally administered MSCs form microtubes and transpose mitochondria toward damaged alveolar type II

cells, which leads to higher alveolar ATP-content and profound protection against lipopolysaccharide-induced acute lung injury (90). In chronic lung injury, therapeutic cells were able to reduce the alveolar damage as well as the interstitial fibrosis by mitochondrial transfer (91). Data supporting the role of mitochondrial transfer in neonatal chronic lung disease are pending.

Safe, Efficacious, Effective? MSCs in Clinical Studies

These promising laboratory studies have lead to early phase clinical trials exploring the feasibility and safety of MSCs in various pulmonary diseases (**Table 1**). Chang et al. recently completed the first phase I dose escalation study using allogeneic human umbilical cord blood-derived MSCs in 9 preterm infants at risk of developing BPD (5). They administered 1×10^7 or 2×10^7 MSCs derived from the cord blood of healthy-term infants intratracheally and observed no serious adverse events or acute toxicity of the cells. Currently, several follow-up studies evaluating the long-term effects of the administered cells are listed on www.clinicaltrials.gov, and a placebo-controlled phase II trial (NCT01828957) is recruiting patients.

Clinical studies with MSCs are warranted. Obviously, MSC therapy in the neonatal population requires extremely careful risk-benefit considerations. Lessons learned from large, placebo-controlled phase III clinical trials using MSCs in steroid-refractory graft-versus-host disease (GvHD) (93) suggest that despite very promising results in animal models and phase I and II studies (94) current MSC preparations have no predictable therapeutic effect. Therapy with MSCs is complex and influenced by more factors than other cellular therapies, such as blood transfusions or hematopoietic stem cells for bone marrow transplantation.

MSCs – a Pharmaceutical Product in the Making

A major problem for clinical trials is the heterogeneity of the cell population termed MSCs. The markers and features defining an MSC are still evolving. As outlined previously, the cells characteristics, such as surface marker-, protein- and gene expression vary with the source, isolation, culture and expansion methods and donor age (35). Virtually every laboratory established (and patented) its own protocols for isolation and culture of MSCs from various sources, which makes it difficult to compare even the results of pre-clinical studies (95).

For clinical trials, a defined, clinical-grade cell product is required. As of now, over 80% of the MSCs used in clinical studies are expanded in media containing fetal bovine serum (FBS) (42), a crude and undefined mixture of growth factors and various bovine proteins. Beyond the unknown influences of various FBS preparations on the therapeutic effect of MSCs, even the potential risk of a pathogen transmission (viruses, prions) makes cells cultured with FBS not optimal for a clinical therapy (96). Based on the MSCs source, many other products used during the isolation process – including enzymes and growth factors – also derive from animal origins. Ideally, a product suitable for administration to a critically ill patient should be produced under current good manufacturing practice (cGMP)-conditions using defined xenogenic free chemicals.

Furthermore, it is crucial to accurately monitor growth and aging of MSCs *in vitro*. It is known that MSCs age during *ex vivo* expansion and that this influences biological properties of the

TABLE 1 | MSCs in clinical trails for pulmonary diseases.

Condition	Phase	Design	Number of participants	Cell origin	NCT ID
Adult ARDS	I	Open	10	bm-msc (allo)	NCT02215811
	I	Randomized, double-blind	9	bm-msc (allo)	NCT01775774
	II	Randomized, placebo-controlled, double-blind	60	bm-msc (allo)	NCT02097641
	I	Randomized, placebo-controlled, double-blind	20	at-msc (allo)	NCT01902082
Air leakage after lung resection	I/II	Open	10	msc N/S (auto)	NCT02045745
Asthma	I/II	Open	20	cdm-uc (allo)	NCT02192736
BPD	I‡ (5)	Open	9	ucb-msc (allo)	NCT01297205
	I	Open	12	ucb-msc (allo)	NCT02381366
	II	Randomized, placebo-controlled, double-blind	70	ucb-msc (allo)	NCT01828957
COPD	II‡ (92)	Randomized, placebo-controlled, double-blind	62	bm-msc (allo)	NCT00683722
IPF	I	Open	18	bm-msc (auto)	NCT01919827
	I‡	Open	8	pla-msc (allo)	NCT01385644
	II	Randomized, open	60	at-msc (auto)	NCT02135380
BOS after lung transplantation	I	Open	9	bm-msc (allo)	NCT02181712
	I	Open	10	msc N/S (allo)	NCT01175655
Pulmonary emphysema	I/II	Randomized, open	30	bm-msc (allo)	NCT01849159

Ongoing and completed interventional clinical trails listed on www.clinicaltrials.gov using mesenchymal stem or stromal cells to treat diseases of the lung.
‡Completed trials are marked with a diesis.
pla-msc, placenta-derived MSCs; bm-msc, bone marrow-derived MSCs; ucb-msc, umbilical cord blood-derived MSCs; at-msc, adipose tissue-derived MSCs; cdm-uc, conditioned media from umbilical cord-derived MSCs; msc N/S, source of cells not specified; allo, allogenic cells; auto, autologous cells.

cells (97). Different methods to determine the age of MSCs have been utilized. Most investigators and companies producing MSCs determine the passage number, an easy but very inaccurate parameter influenced by many factors (98). Therefore, it is not possible to determine if insufficient clinical effects are caused by real therapy failure or just by the fact that senescent therapeutic cells have been administered. A better way than counting passages might be the implementation of cumulative population doubling measurements (99) and biochemical assays, such as telomere attrition or β-galactosidase activity (100).

Prolonged culture of MSCs may also lead to genetic instabilities (101, 102). The spontaneous malignant transformation of MSCs observed in long-term culture experiments (103) has been proven to be an *in vitro* contamination artifact (104). However, the risk of tumorigenicity in MSC-based therapies is still under discussion (99). A direct tumor formation seems unlikely, as MSCs do not engraft. Indeed, in rats receiving MSCs for BPD no tumor masses were seen 6 months after therapy with the cells (16). The risks of increased tumor formation by long-term immunosuppression (99) or the previously discussed stem cell-stimulating effects remain unclear. A first meta-analysis of clinical trials using MSCs showed no increased tumor risk in over 1000 patients after 3–60 months after treatment (105). But as with every drug, definitive data regarding these issues can only be acquired in large clinical trials.

While MSCs are immune-privileged and as such enable allogeneic cell therapy, autologous cell therapy has also been advocated for. Autologous therapy may be associated with lower ethical and technical boundaries than therapy with allogeneic cells. Conversely, the autologous approach is logistically more challenging as it requires the manipulation of a fetal tissue (cord blood, cord stroma . . .) *ex vivo*. Therefore, each product will need to be subjected to a rigorous sterility and quality testing, which takes time, financial, and human resources as opposed to a ready-to-use off-the-shelf allogeneic cell product. It is also not yet clear for which preterm infant an autologous cell product should be processed. Furthermore, the autologous approach may not always be possible (outborn) or potentially deleterious (severe chorioamnionitis). These considerations will mature over time as knowledge and manufacturing technologies advance, allowing us to rationally determine the best possible cell product.

The Quest for a "Potency Assay"

One fundamental problem hampering the widespread use of MSCs in clinical trials is the absence of valid assays to assess their quality or "therapeutic potential" prior to usage.

In applications were the anti-inflammatory effects of MSCs are predominant (like GvHD), tests assessing the immunosuppressive potential of the therapeutic cells may overcome this obstacle (100, 106). In brief, MSCs are co-cultured with mitogen-stimulated allogeneic lymphocytes. They suppress the induced proliferation of the inflammatory cells to various extends via paracrine effects following direct cell-cell interaction. A simple automated cell count assesses the "therapeutic potential" of the MSC-population in this setting. An even faster and easier method uses the interleukin-10 stimulated expression of a specific subtype of the HLA-receptor complex (HLA-G) on the surface of MSCs (107) to assess their immunosuppressive potential. By now, it has not been validated if cells with higher anti-inflammatory potential *in vitro* lead to better therapeutic effects *in vivo* (100).

The situation for multi-factorial diseases affecting the lung – such as BPD – is, however, more complicated. As outlined previously, the mechanisms behind the beneficial effects of MSCs in BPD are complex and involve cytokines, the direct or paracrine interaction with resident cell types and maybe the transfer of mitochondria or exosomes. Therefore, the generation of such a simple functional assay is far ahead. *In vitro* approaches might involve the ability of MSCs to support the generation of alveolospheres out of murine alveolar type II cells in 3D organoid culture systems (108). The assessment of strain resistance in alveolar epithelial cells co-cultured with MSCs *in vitro* might be another interesting approach. Nevertheless, all these approaches remain far from an easy, fast, cheap, and reliable potency assay.

In summary, MSC therapies are promising and clinical conditions, such as BPD, urge for efficient treatment strategies. However, MSC therapies also represent a disruptive technology and for now, not a single trial investigated MSC products in man that met all current regulatory or cGMP criteria (95, 109). A safe and high-qualitative cell product to use in trials is still missing. As outlined recently in a position paper by Wuchter et al. (100), standardization and rigorous quality control of the production process is the *conditio sine qua non* for successful clinical testings using MSCs. If the product does not fulfill these criteria, how should we interpret the clinical results? Every disruptive technology is imperfect at the beginning and needs to evolve with experience and time. But it is imperative to do due diligence and obtain the best possible cell product before testing it in our most vulnerable patients.

No Vessels, No Lung Growth: Progenitor Cells from the Endothelial Lineage

Simplification of the pulmonary vasculature is a hallmark of BPD (110), and angiogenesis is crucial for normal postnatal alveolar development (111). Hyperoxia-induced lung injury can be attenuated by increasing the pulmonary supply of angiokines like VEGF in rodents (111, 112). Accordingly, if vascular growth factors and lung angiogenesis contribute to the integrity of the lung, then vascular progenitor cells are appealing candidate cells likely to be involved in the same mechanisms.

After their first description as circulating cells in the peripheral blood by Asahara et al. (113), endothelial progenitor cells have been shown to promote the repair of damaged blood vessels in various disease models [reviewed by Mund and colleagues (114)]. They are further investigated as biomarkers of cardiovascular diseases [reviewed by Sen et al. (115)]. EPCs harbor the potential to form tube-like structures on matrigel matrices *in vitro*, home to ischemic sites *in vivo*, and augment angiogenesis by paracrine effects (116).

However, the population termed EPCs is not homogeneous, and the exact origin and definition of these cells remain unclear. A direct relationship of EPC subsets to the myeloid progenitor line has been described (117). Two groups provided evidence for a hierarchy within circulating EPCs and identified a specific subset named blood outgrowth endothelial cells (BOEC) (118) or

endothelial colony forming cells (ECFCs) (119). This population, further referred to as ECFCs, is thought to contain the therapeutically active progenitor cells of the endothelial lineage (117). In contrast to the global EPC population, ECFCs lack expression of CD133 and CD115, exert high-clonal proliferative potential and harbor the ability to form vessels *de novo* when transplanted into immunodeficient SCID-mice [recently reviewed by Basile and Yoder (116)].

Endothelial Progenitors in BPD

Using a mouse model of BPD, Balasubramaniam et al. described that hyperoxia-induced lung damage depletes circulating EPCs and bone marrow-derived angiogenic cells (BMDACs) (120). Administration of BMDACs from healthy mice rescues the alveolar and vascular structure after O_2 injury (31).

The role of circulating endothelial progenitors in the pathogenesis of BPD was further confirmed in studies with human infants. Borghesi and colleagues described that high numbers of ECFCs in the cord blood of preterm babies are associated with a lower risk to develop BPD (121). Interestingly, the blood counts of non-ECFC endothelial progenitors fail to predict or correlate to any disease associated with preterm birth (122), further substantiating the role of circulating ECFCs. Baker et al. also reported the association between low-ECFC counts and the development of BPD. They further showed that a decreased ratio between circulating progenitor cells with pronounced *in vitro* angiogenic potential (CPC) and those without (non-angiogenic, non-CPC) predicts the development of moderate or severe BPD (123). Moreover, ECFCs isolated from preterms are more prone to oxidative stress than cells from term infants (124). CdM from cord blood-derived ECFCs obtained from term infants promotes growth of the pulmonary vasculature, but fails to promote alveolar septation in bleomycin-induced lung injury (125).

The lung also harbors its own resident progenitor cells with vasculogenic capacity (30, 126, 127). Human fetal and neonatal rat lungs contain ECFCs with robust proliferative potential, secondary colony formation on replating, and *de novo* blood vessel formation. Exposure to hyperoxia *in vitro* and *in vivo* impedes ECFC function as exemplified by decreased proliferation, clonogenic, and angiogenic capacity. In experimental chronic hyperoxic lung injury in rats, administration of human cord blood-derived ECFCs restored resident lung ECFC colony- and capillary-like network-forming capabilities, lung function, alveolar and lung vascular growth, and attenuated pulmonary hypertension. At 10 months post-ECFC therapy improvement in lung structure, exercise capacity, and pulmonary hypertension persisted without signs of adverse effects (30). Comparable to MSCs, the benefit seems to be mediated by a paracrine effect since cell engraftment was minimal and CdM from ECFCs exerted similar therapeutic benefit to whole cell therapy.

Room for a Clinical Application?

As of February 2015, no clinical trials using endothelial progenitor cells or their CdM as therapeutic agents in BPD are listed on www.clinicaltrials.gov. In the past, Wang et al. conducted two clinical trials in adult patients suffering from idiopathic pulmonary hypertension (NCT00641836 and NCT00257413). They used a heterogeneous preparation of autologous endothelial progenitors and demonstrated safety and feasibility as well as significantly increased exercise capacity and reduced pulmonary blood pressures 12 weeks after intravenous administration (128). A Canadian phase I study using EPCs transfected with endothelial nitric oxide synthase (eNOS) in seven patients has recently been completed (NCT00469027); final results are pending.

EPCs for therapeutic purposes could be isolated from easily accessible peripheral blood or cord blood without the ethical problems raised by a bone marrow puncture to obtain BMSCs. With the SCID-mouse transplantation assay, an excellent and reliable method assessing the functional capacity of ECFCs is available (117). However, the relatively complicated isolation and expansion process requires sophisticated (and expensive) media as well as many manual steps including the individual lifting of emerging colonies (117, 119).

As of today, no large-scale production technique has been developed to reliably isolate the quantities of cells required for clinical studies. Compared to MSCs, less is known about the behavior of EPCs or ECFCs *in vivo* and *in vitro*. Nevertheless, given the importance of angiogenesis for a large variety of diseases, cell-based vascular therapies will rapidly develop as our understanding of EPC biology advances in parallel with our knowledge in bioengineering and cell manufacturing.

Not Stem Cells, Still Therapeutic: Amnion Epithelial Cells

Cells from the human amniotic epithelium (AECs) represent the third cell population that has been explored in experimental BPD. The amniotic membrane is widely used as an effective and low-immunogenic material to patch large skin defects (129). This tissue contains epithelial cells with distinct regenerative (130) and an anti-inflammatory potential (131) comparable to MSCs (132). AECs further possess the potential to differentiate along mesodermal, ectodermal, and endodermal lineages *in vitro* (130) and are considered "stem-like cells" (133).

In 2010, Moodley and colleagues described that i.v. injection of AECs abrogates lung fibrosis and inflammation in bleomycin-challenged immunodeficient mice. Furthermore, the cells homed and engrafted permanently into the damaged lung tissue, acquired the phenotype of alveolar type II cells, and started producing surfactant (133). In immunocompetent animals, similar effects without cell engraftment were observed (134). The potent anti-inflammatory and anti-fibrotic effects led to studies in fetal sheep with intraamniotic LPS-induced lung injury. Here, i.v. administration of AEC to the unborn lamb led to reduced lung inflammatory cytokines without significant improvements on lung structure (29).

In a study using *in utero* ventilation of fetal sheep to induce BPD-like changes in lung histology, Hodges et al. demonstrated significant improvements of the lung structure after combined i.v. and intratracheal administration of AEC during the ventilation procedure. Engraftment and transdifferentiation of AECs into alveolar type I and type II cells were noted. However, these rare events did not contribute to the overall impact of AECs in this animal model (28).

Currently, no clinical trials using AECs in pulmonary diseases are listed. However, AECs are investigated in a clinical trial for ocular limbal stem cell deficiency (135). Large quantities of the AECs can easily be produced from birth-associated tissues. But by now, no clear definitions and characterization regimen to define amniotic epithelial cells exist (136). Cells used in pre-clinical studies represent heterogenous populations, expressing a large variety of surface markers labeling pluripotent (SSEA-4), epithelial (cytokeratin-7, EpCam), and mesenchymal cells (CD73, CD90, CD166, among others) (29, 133). Nevertheless, first encouraging steps toward controlled, cGMP-conform isolation methods have been undertaken (137).

Conclusion

Cell therapies represent the next paradigm shift in medicine. Unlike previous therapeutic game-changers, such as small molecules and biologics, cells are part drug and part device, which can sense diverse signals, interact with their environment, integrate inputs to make decisions, and execute complex response behaviors (138). These unique attributes of stem cells have been harnessed for organ regeneration. In the developing lung, various cell types including MSCs, EPCs, and AECs harbor the fascinating potential to provide pleiotropic therapeutic agents to protect from and restore lung damage. These cells are thus ideally suited not only for the treatment of a multi-factorial disease, such as BPD, but also for other complications of extreme prematurity.

Phase I trials with MSCs have already started and while the time is ripe for carefully designed early phase clinical trials, more progress is required to better understand the mechanisms of action and to optimize cell products. As with all disruptive technology, there is a steep learning curve in the beginning and the first product may be imperfect. Early on, common reference standards of the isolation and manufacturing process should be established to ensure uniformly high quality, effective and practical cell products. This will be crucial not only to ensure success of stem cell clinical trials but also to interpret and compare these trials. Finally, establishment of registries of all treated patients are imperative to ensure long-term follow-up.

Three excellent reviews further addressing the obstacles of bench-to-bedside transition of current stem cell therapeutics have been written by D. Prockop, S. Prockop, and I. Bertonello (95), G. Daley (139) and M. Fischbach, J. Bluestone, and W. Lim (138).

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REVIEW
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Animal models, learning lessons to prevent and treat neonatal chronic lung disease

*Alan H. Jobe**

Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, University of Cincinnati, Cincinnati, OH, USA

Bronchopulmonary dysplasia (BPD) is a unique injury syndrome caused by prolonged injury and repair imposed on an immature and developing lung. The decreased septation and decreased microvascular development phenotype of BPD can be reproduced in newborn rodents with increased chronic oxygen exposure and in premature primates and sheep with oxygen and/or mechanical ventilation. The inflammation caused by oxidants, inflammatory agonists, and/or stretch injury from mechanical ventilation seems to promote the anatomic abnormalities. Multiple interventions targeted to specific inflammatory cells or pathways or targeted to decreasing ventilation-mediated injury can substantially prevent the anatomic changes associated with BPD in term rodents and in preterm sheep or primate models. Most of the anti-inflammatory therapies with benefit in animal models have not been tested clinically. None of the interventions that have been tested clinically are as effective as anticipated from the animal models. These inconsistencies in responses likely are explained by the antenatal differences in lung exposures of the developing animals relative to very preterm humans. The animals generally have normal lungs while the lungs of preterm infants are exposed variably to intrauterine inflammation, growth abnormalities, antenatal corticosteroids, and poorly understood effects from the causes of preterm delivery. The animal models have been essential for the definition of the mediators that can cause a BPD phenotype. These models will be necessary to develop and test future-targeted interventions to prevent and treat BPD.

Keywords: prematurity, lung injury, inflammation, oxidant injury

Edited by:
Anne Hilgendorff, Helmholtz Zentrum München, Germany
Andrew Bush, Royal Brompton Hospital and Imperial College London, UK

Reviewed by:
Gunnar N. Hillerdal, Karolinska University Hospital, Sweden
Michael Adam O'Reilly, The University of Rochester, USA

***Correspondence:**
Alan H. Jobe, Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, University of Cincinnati, 3333 Burnet Avenue, ML#7029, Cincinnati, OH 45229-3039, USA
alan.jobe@cchmc.org

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Introduction

My charge is to evaluate the contributions from animal models that have resulted in better care to prevent and treat chronic lung disease in newborns, which I will refer to as bronchopulmonary dysplasia (BPD). I will focus exclusively on the BPD associated with very preterm birth (1). This review will move between observations in animal models and the clinical syndrome of BPD with a skeptical eye toward how results from models have translated to clinical outcomes. The cynic might say that there has been no progress in the care of infants with BPD based on a recent systematic review of randomized controlled trials (RCT) for the prevention of BPD (2). Of 47 RCTs for drug therapies, only eight showed benefits for five agents (vitamin A, caffeine, dexamethasone, inositol, and clarithromycin) and none of the 47 trials were registered for an Investigational New Drug for BPD. This depressing perspective is in part the result of the lack of trial data to document the continuing improvements in respiratory support and general clinical care that have strikingly

decreased mortality for preterm infants since 1967. For example, the two major interventions of surfactant and antenatal corticosteroid treatments decrease the incidence and severity of respiratory disease syndrome (RDS) and decrease mortality after very preterm birth (3, 4). The collateral damage of increased survival is that more high infants are at risk of developing BPD. Antenatal corticosteroids and surfactant change the population of infants at risk for BPD but do not decrease the incidence of BPD. As the disease has evolved to primarily occur in the most immature infants, the concepts about pathophysiology depend more on studies of developing lungs in preterm and term animals. This review will focus primarily on models of BPD in term newborn and preterm animals. My perspective of the pathophysiology of BPD is that the programs required for normal lung development from the saccular to the alveolarized lung are clashing with prolonged exposures to lung injuries from oxygen toxicity, mechanical stretch, and inflammation and simultaneously with repair programs to yield a most complex and varied pathophysiology (**Figure 1**). Each of the three elements in the equation – development, injury, and repair – is crucial to anticipating how an animal model might contribute to understanding the pathophysiology of BPD and to the testing of treatment strategies.

An Historical Perspective on Animal Models of BPD

Oxygen and BPD

Soon after the description of BPD as a progressive lung injury in ventilated premature infants exposed to at least 150 h of 80–100% oxygen (5), Northway's group reported that newborn mice survived for weeks in 100% oxygen with only a small increase in lung wet weights (6, 7). In contrast, adult mice died within several days and had heavy and inflamed lungs. The histopathology in the newborns demonstrated type II cell proliferation and a hypertrophic bronchiolar epithelium. This increased survival of newborns occurred despite low levels of the antioxidant enzymes that ostensibly should put the newborn lung at increased risk of injury (8). Nevertheless, the most frequently used animal model for evaluating therapies for BPD is the newborn rodent – rats or mice – exposed to various amounts of oxygen for periods of days to weeks as reviewed by Hilgendorff et al. (9).

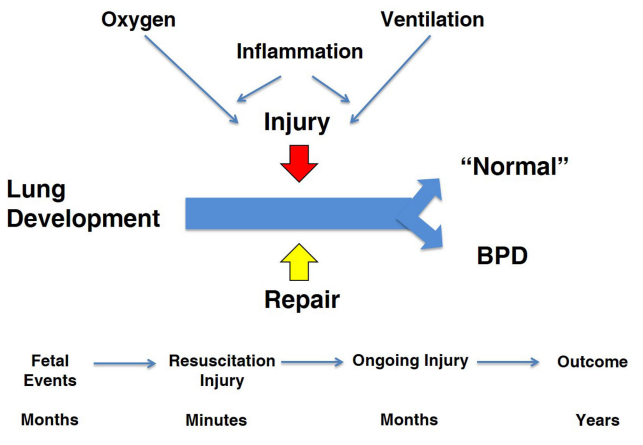

FIGURE 1 | Overview of "big events" in pathophysiology of BPD. The three major programs that are active during the course of BPD are lung development, injury, and repair. The major injuries are from oxygen and ventilation-associated inflammation. The time line for the fetus and infant occurs over months to years, which contrasts to studies in term rodent models.

These rodent models are attractive as they are reproducible, readily available, and relatively inexpensive. Oxidant injury of the newborn rodent lung delays saccular septation to alveoli, increases mesenchymal cellularity, causes a persistent inflammatory response, blocks capillary formation in the distal lung, and causes pulmonary hypertension (10). These changes are similar to the abnormalities described for the lungs of infants who have died of BPD after several months of life (11). Oxygen toxicity models are deceptively simple and are assumed to capture the key elements of the pathophysiology of BPD. However, multiple variables of relevance to BPD can modulate oxygen toxicity responses. As noted above, high concentrations of oxygen kill adult rodents within about 3 days but newborns can survive for weeks in 100% oxygen (6). This transition to oxygen sensitivity occurs after about 30 days of age in the rat (12). Calorie restriction of pregnant rats or newborn rabbits increases the oxygen sensitivity of the lung (13). In adult rats and mice, a variety of exposures prior to an exposure to 100% oxygen can greatly decrease mortality from oxygen – the phenomenon of preconditioning. For example, adult rats exposed to 100% oxygen for 48 h and then returned to air for 24 h prior to a second exposure to 100% oxygen survived for 7 days (14). A preexposure of adult rats to endotoxin survive for >3 days in oxygen (15). Fetal sheep exposed to antenatal corticosteroids or to intra-amniotic endotoxin have large increases in antioxidant enzymes in the fetal lungs (16, 17). Many infants at risk of BPD have been exposed to intra-amniotic infection, corticosteroids, and nutritional deficits. Further, hypoxia is a potent preconditioning exposure (18). The assumption generally is that extremely low birth weight (ELBW) infants are very sensitive to oxygen toxicity. However, the animal model data suggest that ELBW infants may have quite variable sensitivities to oxygen at different times during their clinical course because of their antenatal and postnatal exposures. There is no information about oxygen sensitivity in ELBW infants or how that might be assessed (19).

Ventilation and BPD

In 1960s, mechanical ventilation of large preterm infants with RDS with primitive ventilators and no positive end expiratory pressure (PEEP) resulted in mortality rates of about 70% worldwide. In that context, Northway and colleagues (5) hypothesized that the combination of very high and prolonged oxygen exposure, together with mechanical ventilation, caused the new syndrome of BPD. The initial animal models explored the oxygen exposure component of the injury and no clinical progress was made with ventilation strategies until Gregory and colleagues in 1971 (20) described continuous positive airway pressure (CPAP) to open the atelectatic and surfactant deficient lungs of infants with RDS which improved oxygenation and survival. CPAP was quickly designed into infant ventilators to provide PEEP. The respiratory care of the larger infant with RDS was transformed within several years without much in the way of clinical trials or animal-based

research. However, smaller and more immature infants now survived with a new variant of BPD (11).

Animal models then became critical for understanding the link between mechanical ventilation and injury of the preterm lung. Robertson and colleagues developed techniques for short-term ventilation of preterm rabbits, primarily to test physiological responses to surfactant treatments (21). They described severe bronchiolar epithelial disruption within minutes of ventilation of surfactant deficient preterm rabbits (22). The epithelial injury resulted in airspace flooding with intravascular proteins, which could be prevented with surfactant treatment (23). Escobedo et al. (24) and Coalson et al. (25) ventilated premature baboons with oxygen and described chronic BPD-like lung changes as a model to explore the pathogenesis of BPD. This group related the progression of "diffuse alveolar damage" from the combined exposures of oxygen and mechanical ventilation to similar studies in adult baboons with adult respiratory distress syndrome (ARDS) (26). The exudative response to the injury was delayed and blunted in the preterm relative to the adult, a result consistent with less injury from oxygen. However, airway injury was more severe in the preterm lungs. The review of BPD by O'Brodovich and Mellins (27) in 1985 identified oxygen as the primary instigator of BPD, although mechanical ventilation was recognized as a contributor.

Concurrently, with extensive adult animal model research on the mechanical causes of lung injury (28, 29), short-term studies with preterm sheep demonstrated that the preterm lung was exquisitively sensitive to lung injury from volutrauma (30). With the initiation of ventilation of the fluid-filled lung of the preterm lamb with just six very large tidal volumes of 35–40 ml/kg, the preterm lung was injured such that there was no response to surfactant treatment (31) (**Figure 2**). Tidal volumes of 20 ml/kg for 30 min of ventilation resulted in better compliance and gas

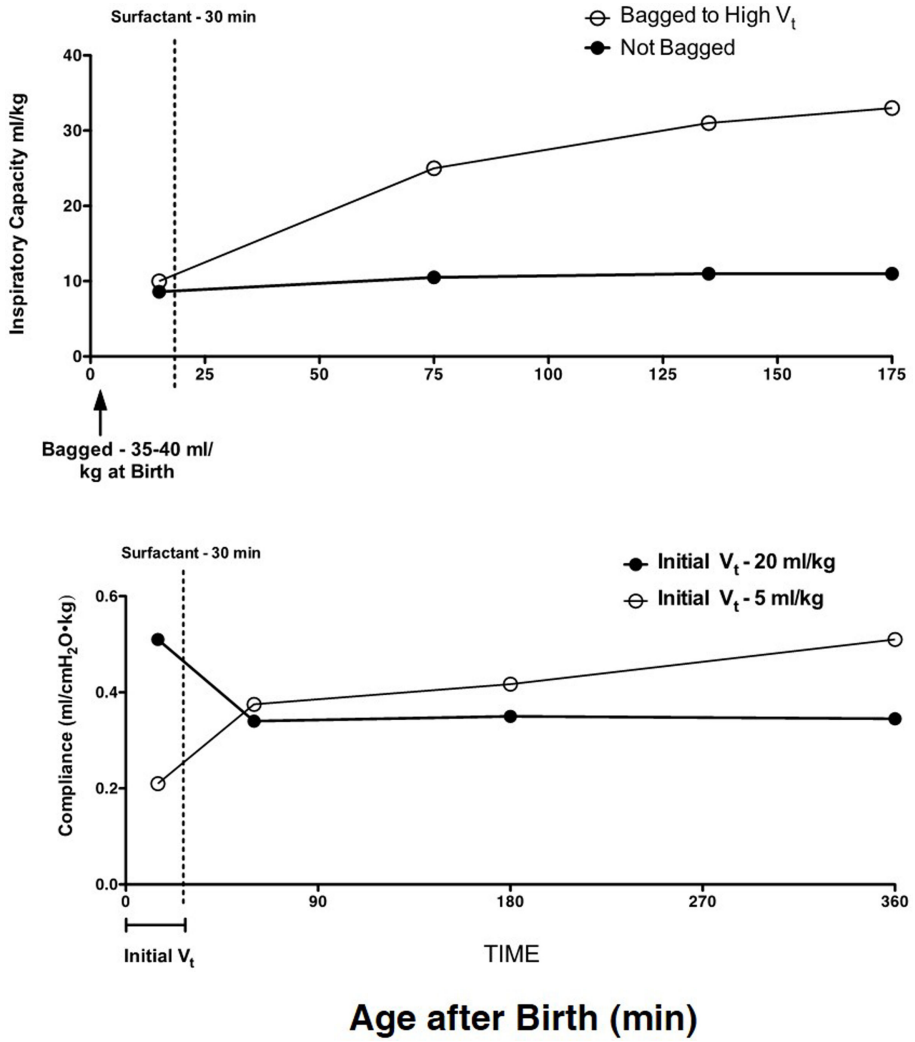

FIGURE 2 | High tidal volumes inure the preterm lung. Six large tidal volumes given by hand at delivery injure the preterm lamb lung and prevent a response to surfactant given at 30 min of age. Redrawn from data in Ref. (31). B. An initial tidal volume of 20 ml/kg results in a high compliance in preterm lambs relative to a low tidal volume of 5 ml/kg, but at 30 min of age, the surfactant response is lost with the high tidal volume. The low tidal volume to 30 min of age allows the lungs to respond to surfactant. Redrawn from data in Ref. (32).

exchange than tidal volumes of 5 or 10 ml/kg, but the higher tidal volume injured the lungs (32). This result is similar to the outcomes of tidal volume ventilation of ARDS patients: a higher tidal volume resulted in better gas exchange in the short-term but more mortality from lung injury (33).

The association of both mechanical ventilation and oxygen with BPD in infants and premature animal models is inevitable as both the infants and the animals generally require both to survive. Using short-term fetal mechanical ventilation with air in sheep, O'Reilly et al. (34) reported injury and bronchiolar remodeling over 7 days. Hillman et al. (35) of ventilated exteriorized fetal sheep for 15 min with 100% nitrogen or 100% oxygen and returned the animals to the uterus for 3 h. Expression of response genes and cytokines demonstrated large injury responses but no differences between the nitrogen- and oxygen-exposed lungs. With chronic ventilation of preterm lambs for 3 weeks using oxygen concentrations <30%, Bland et al. (36) reported increased and abnormally distributed elastin and reduced expression of growth factors that regulate alveolarization. Innovative experiments with ventilated newborn mice for 7 h with 40% oxygen or room air disrupted elastin synthesis and increased apoptosis (37). A subsequent study with 24 h air ventilations of newborn mice demonstrated increased apoptosis and an inhibition of alveolarization and microvascular development, the key findings in BPD (38). These effects were remarkable in that the ventilation induced the BPD phenotype quickly and without a concurrent oxygen exposure or inflammation. In contrast to newborn mice, room air may be "hyperoxic" for the very preterm human lung and is thus an unavoidable exposure. Nevertheless, these experiments with mice in the saccular phase of lung development are proof of principle that mechanical ventilation alone can cause a BPD phenotype.

Lung injury from mechanical ventilation has progressed from considerations of pressure (barotrauma) to focused analyses of tidal volumes (volutrauma), atelectotrauma (ventilation from low lung volumes), and biotrauma (proinflammatory mediators), primarily using adult animal models (39, 40). Strategies to decrease ventilator-induced lung injury (VILI) are major components of efforts to improve outcomes for patients with ARDS (39). VILI was recently compared in adults, children, and with an analysis of animal models (41). Newborn rodents have larger lung volumes [functional residual capacity (FRC) and total lung capacity (TLC)] than adult rodents, which seems to translate to less injury at a given tidal volume in the newborn than for adult animals (42, 43). However, the term newborn rodent is not a good model for the preterm infant at risk of BPD. The preterm infant has a low FRC and a low TLC compared with the term infant or the adult human (30). Further, the surfactant-deficient preterm lung requires mean airway pressures > 20 cmH_2O to maximally recruit lung volume (44). The preterm lung is easier to overstretch because the chest wall is compliant and the lung collagen matrix is insufficient to limit stretch of both the airways and the parenchyma (45). The lung volume available between FRC and TLC for safe mechanical ventilation may be as low as 15 ml/kg. Thus, with an unstable FRC and a poorly defined TLC, VILI can easily occur with mechanical ventilation. The animal models most relevant for understanding how ventilation injury contributes to BPD are preterm sheep or primates who can be ventilated for prolonged periods so that injury progression can be evaluated.

Inflammation and BPD

A common theme since the early days of BPD research is the association between inflammation and BPD. Airway aspirates from infants with early lung injury that progresses to BPD contain increased numbers of inflammatory cells and proinflammatory cytokines (46, 47). As in adult animal models with high oxygen exposures alone, newborn rodent lungs respond to injury with recruitment and activation of inflammatory cells and increased proinflammatory cytokines in alveolar washes (10). Similarly, stretch-mediated injury causes a brisk proinflammatory response in preterm sheep (35). Very preterm baboon lungs developing BPD from oxygen and mechanical ventilation have greatly elevated proinflammatory cytokines for the 4-week period of study (48) (**Figure 3**). The preterm fetal sheep lung has a large inflammatory response to just 15 min of stretch from ventilation with nitrogen using high (15 ml/kg) or to relatively normal tidal volumes of 6 ml/kg (49, 50). The inflammatory response is less with the lower tidal volume, with antenatal corticosteroid treatment, with surfactant treatment prior to ventilation, or with ventilation with PEEP (51–53). However, even combinations of approaches will not eliminate an inflammatory response with ventilation of the preterm fetal lung. Further, the 15 min lung stretch injury is greatly amplified if the preterm fetus is subsequently delivered and ventilated for brief periods. These experiments in baboons

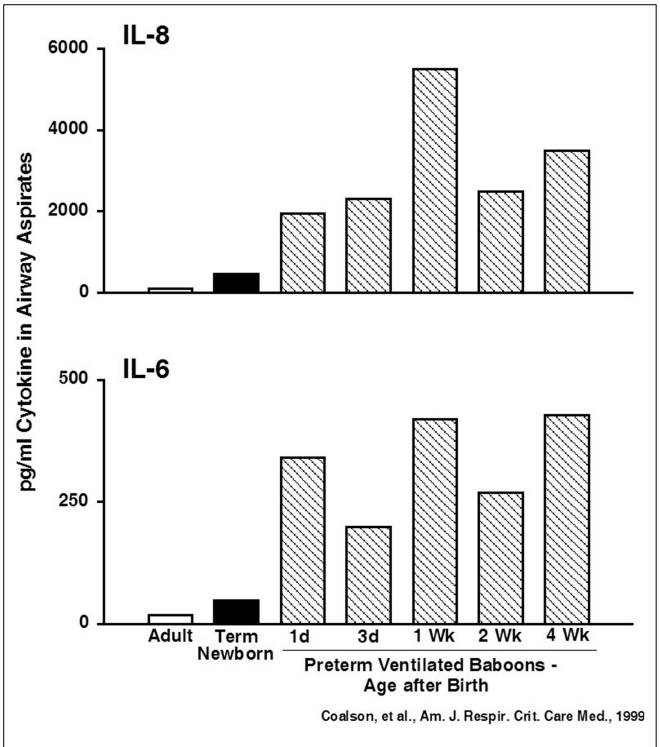

FIGURE 3 | Cytokines are persistently elevated in airway samples from ventilated preterm baboons. IL-6 and IL-8 were increased from day 1 to week 4 in preterm baboons progressing to BPD. Data redrawn from Coalson et al. (48).

and sheep demonstrate that the preterm lung can generate rapid and very prolonged inflammatory responses to care elements that most ELBW infants need for survival.

However, the clinical environment is much more complex as more than 50% of ELBW infants will have been exposed to infection/inflammation from chorioamnionitis prior to delivery (54). An increased risk of BPD may be associated with fetal exposure to histologic chorioamnionitis, although the increased risk has been inconsistent in the multiple evaluations of the association (55). Watterberg et al. (56) made the intriguing observation that preterm infants exposed to histologic chorioamnionitis had a decreased risk of RDS but an increased risk of BPD. The infants exposed to chorioamnionitis had increased amounts of inflammatory mediators and cells in airway aspirates collected shortly after birth. Therefore, the lung inflammation proceeded the delivery and subsequent exposures to oxygen and ventilation. The difficulty with a consistent interpretation of the clinical data is that chorioamnionitis is caused by organisms ranging from commensals to pathogens, the fetal exposure is for various periods prior to delivery, and the intensities of the exposures are variable (57).

Animal models have been effectively used to characterize the ranges of fetal lung responses to inflammatory exposures. Bry et al. (58) first reported that intra-amniotic injections of interleukin 1 (IL-1)-induced lung maturation in preterm fetal rabbits as measured by increased mRNA for surfactant proteins, increased surfactant lipids, and improved pressure–volume curves. Intra-amniotic injections in fetal sheep of IL-1 or *E. coli* endotoxin lipopolysaccharides (LPS), but not IL-6, IL-8, or TNF-α, induce chorioamnionitis and early lung maturation as measured by large increases in pressure–volume curves and lung mechanics and gas exchange within 5 to 7d (59–61). The improvement in lung performance is proceeded by increases in mRNA for the surfactant proteins and then by increases in surfactant lipids (62). The clinical phenotype consistent with these early maturational effects would be a decrease in the severity of RDS, which should decrease the risk of BPD.

The maturation response to fetal exposure to chorioamnionitis induced by IL-1 or LPS is proceeded by modest inflammation characterized by recruitment of activated granulocytes to the fetal lung parenchyma and airspaces, high levels of proinflammatory cytokine expression, increased apoptosis, and subsequent cell proliferation (63). This injury response includes a transient inhibition of alveolar septation and causes pulmonary vascular injury with increased smooth muscle in vessel walls and decreased mediators of vascular development (64, 65). The fetal lung has more functional maturation and more antioxidant enzymes but with septation and microvascular injuries consistent with a mild BPD phenotype. Despite prolonged exposure to intrauteral inflammation, infants have not been described as having BPD at birth. This observation is consistent with the animal models, as repetitive or continuous exposures for 28 days to intra-amniotic LPS do not cause progression of the septation and microvascular injuries (66). In fact, the preterm lung heals despite continued exposures to proinflammatory agonists. The proinflammation is downregulated soon after exposure to agonists, and the fetal lung "tolerizes" in that subsequent inflammatory mediator exposures do not incite inflammation in the fetal lung (67). LPS is the agonist most used in animal models to evaluate effects on the fetal lung. LPS inhibits structural development of the fetal mouse lung via TLR4 receptors and NF-kB activation with suppression of FGF-10 (68, 69). Mediators such as TGF-β1 and the SMADS are induced while others such as connective tissue growth factor (CTGF) and caveolin-1 are suppressed, demonstrating multiple effects on LPS on the fetal lung that may contribute to the changes of BPD (70, 71).

Multiple different organisms and polymicrobial infections have been associated with histologic chorioamnionitis, but different clinical presentations have not been associated with the types of infection (57). In the fetal sheep or monkey, quite distinct responses of fetal exposures to organisms and mediators can be demonstrated. Intra-amniotic LPS or IL-1 causes limited but very complex inflammatory responses that yield the phenotypes of lung maturation, a mild BPD, and immune tolerance (67, 72). Ureaplasma species are the most frequent organisms associated with preterm labor and they may cause chronic and indolent colonization (infection?) and have been associated with BPD (73). Ureaplasma readily colonize the amniotic fluid of fetal sheep and cause a generally mild but variable chorioamnionitis that can persist for months (74). The fetal lung has a very modest inflammatory response with low-grade cytokine expression and a small increase in granulocytes (75). However, the induced lung maturation and microvascular changes are reminiscent of LPS effects (76, 77). In contrast, intra-amniotic *Candida albicans* causes a modest chorioamnionitis but a severe consolidating and necrotizing pneumonia with a progressive lethal fetal inflammation within about 3 days (78). These varied responses in animal models provide the insight that chorioamnionitis may initiate BPD by decreased septation and microvascular injury in fetal life but may also protect from BPD by inducing lung maturation and increasing antioxidant enzymes. As recently reviewed by Wright and Kirpalani (79), attempts to target inflammation have not translated to therapies for BPD.

Animal Models to Evaluate Therapies for BPD

Overview

The major elements of BPD that have been targeted for therapies are the arrest of alveolarization, the blunted microvascular development, and the inflammation that is generally considered a common path to the septation and vascular abnormalities. The concept that inflammation was a common mediator for the progression of BPD resulted from the observations that inflammation accompanied oxygen toxicity and ventilation-mediated injury in newborn and adult animals and that overexpression of most any proinflammatory cytokine in newborn mice lungs resulted in inflammation and septation abnormalities (11). Early examples of a BPD phenotype were overexpression of TGF-α, IL-11, and IL-6 (80–82). More recently, overexpression of IL-1β or bioactive TGF-β1 in airway epithelial cells in newborn mice were proposed as models of BPD (83, 84). The experimental strategy to assess a therapeutic benefit has generally been to cause a BPD-like injury with oxygen exposure of newborn rodent lungs and to assess agonists or antagonist effects on inflammation and changes in septation and/or microvascular development. Primate and sheep

models of combined ventilation and oxygen exposure have been used to better replicate clinical reality and to test interventions such as high-frequency ventilation (HFV) and inhaled nitric oxide (iNO). I will assume that newborn rat and mouse models of oxygen exposure are equivalent and I will not address stem cell therapies. In general, the reports focus on individual mechanisms and pathways, but many of the interventions may have pleotropic (and presently unknown) effects.

BPD Therapy by Blocking Inflammatory Cells

If BPD is caused by chronic inflammation in the developing lung, then blocking the major inflammation factories – the granulocytes and macrophages should be effective. Auten et al. (85) demonstrated that antibodies to the cytokine – induced neutrophil chemoattractant-1 (CINC-1) effectively blocked neutrophil recruitment to the lungs and alveolar washes of 95% oxygen-exposed newborn rats and alveolar architecture was preserved. This observation was extended to demonstrate that blocking the signaling of CINC-1 through its receptor CXC chemokine receptor-2 with a small molecular weight antagonist completely blocked neutrophil influx into the lungs of 60% oxygen-exposed newborn rats (86). Surprisingly, the 60% oxygen exposure, together with blocked neutrophil influx, enhanced lung growth and septation. Treatment of newborn rats with 60% oxygen and IL-1 receptor antagonist blocked neutrophil recruitment to the lungs and improved alveolar septation with less of an effect on the pulmonary vascular injury (87). Neutrophil recruitment to the lungs of fetal sheep exposed to LPS can be effectively blocked by either an anti-CD-18 antibody or by intra-amniotic IL-1 receptor antagonist (88, 89). Either strategy to decrease inflammatory cell recruitment to the fetal lungs prevents both the injury and the lung maturation responses. Strategies to interfere with neutrophil recruitment to prevent BPD have not been tried in infants at risk. Such strategies have not been effective for ARDS and infants have depressed immune responses that would be further compromised by inhibition of granulocyte function.

The normal developing lung contains few mature macrophages until after birth when an increase in GMCSF in the lungs activates the transcription factor PU-1 in immature monocytes to differentiate to mature monocytes (90). However, macrophages become prominent in airway samples of infants with BPD. Monocytes in the lungs of fetal sheep exposed to a 15 min volutrauma injury mature within 24 h to macrophages as do fetal lung monocytes exposed to intra-amniotic LPS (90, 91). Jankov et al. (92) used gadolinium chloride to block macrophage influx into the lungs of newborn rats exposed to 60% oxygen. The treatment decreased macrophages and prevented smooth muscle hypertrophy in pulmonary vessels and prevented pulmonary hypertension but not the septation defect – a partial response at best. As with inhibitors of granulocyte recruitment, macrophage depletion may put the preterm at risk of infection.

Targeting overall lung cell metabolism has not been exploited for a possible therapy for BPD. In newborn mice exposed to 75% oxygen, decreased septation correlated with decreased mitochondrial oxidative phosphorylation (93). Thus, a general cellular bioenergetics failure maybe a fundamental mechanism contributing to BPD. Inflammasome-mediated cytokine injury has not been explored in the developing lung. Mechanical ventilation of adult mice activated inflammasomes with IL-18 release, an effect that was blocked with an anti-IL-18 antibody with reduced lung injury (94). These reports may be relevant to future BPD research.

Antioxidants to Prevent BPD

Increased oxygen exposure is considered a major cause of BPD, and oxygen is the agonist for the newborn rodent models of BPD. Lung antioxidant enzymes are decreased with preterm birth (8), so that antioxidant treatments are logical. Clinically, *N*-acetylcysteine and recombinant superoxide dismutase did not decrease BPD (95, 96). Vitamin A, which may have antioxidant effects, did modestly decrease BPD (97). Consistent with the clinical result, Vitamin A improved septation and alveolar capillary growth in chronically ventilated preterm lambs (98). Vitamin A also modulated elastin metabolism and the expression of vascular growth factors as mechanisms distinct from antioxidant effects. In preterm ventilated baboons given 100% oxygen for 10 days, a small molecular weight catalytic antioxidant improved lung anatomy relative to untreated animals (99). Peroxynitrites are products of oxidant exposure, and a catalyst that decomposes peroxynitrite decreased both the alveolarization and the vascular injuries resulting from exposure of newborn rats to 60% oxygen (100, 101). The animal model research with antioxidants for BPD is not extensive and might be a productive avenue toward a therapy.

Nitric Oxided to Prevent BPD

The animal model literature on the benefits of iNO for the preterm lung is large and positive. Lambs exposed to mechanical ventilation have decreased neutrophil accumulation and edema if exposed to iNO (102). iNO also attenuates pulmonary hypertension and improves lung growth in newborn rats exposed to hyperoxia (103), as does the related compound ethyl nitrate (104). iNO also corrects the lung structural abnormalities and pulmonary hypertension from a bleomycin-induced lung injury in neonatal rats that resembles BPD (105). iNO can improve the lung structural abnormalities caused by blockade of the vascular endothelial growth factor (VEGF) receptor, suggesting iNO effects on growth factor signaling (106). These beneficial effects translated to chronically ventilated large animal models in preterm lambs and baboons. Bland et al. (107) found that iNO protected ventilated lambs from alveolar simplification and increased airway resistance. iNO treatment of preterm ventilated baboons over 14 days improved lung function, improved septation, preservation of lung growth, and normalization of extracellular matrix deposition (108). As a treatment strategy to mimic NO signaling, Ladha et al. (109) treated newborn 95% oxygen-exposed rats with sildenafil to increase cGMP and improved alveolar growth with less pulmonary hypertension. Another approach used by McCurnin et al. (110) was to give estradiol to increase NO synthases, a treatment that improved lung function of preterm ventilated baboons over 14 days, but without clear effects on lung structure or elastin deposition. Despite this extensive literature in both newborn rodents and chronically ventilated sheep and baboons that identify multiple beneficial effects of iNO, multiple clinical trials show no consistent benefit of iNO for the prevention of BPD in preterm infants (111), a most disappointing result.

Growth Factors in the Pathogenesis of BPD

Lung growth from the saccular to alveolar stages of development is regulated by multiple growth factors that may be dysregulated during the course of BPD. In newborn rats, inhibition of VEGF decreases lung septation and microvascular development in parallel (112), a phenotype similar to the oxygen-exposed newborn rodent. Therefore, Kunig et al. (113) exposed newborn rats to 75% oxygen for 14 days and then treated the animals with intramuscular injections of rhVEGF and demonstrated improved recovery of vascular growth and alveolarization relative to controls. A similar result was reported for prevention of the initial oxygen injury in newborn rats (114). The vascular and alveolar development that was inhibited by 95% oxygen did not occur with postnatal adenovirus-mediated VEGF therapy. Optimal treatment included both VEGF and angiopoietin-1 gene transfers to decrease the vascular leakage caused by the increased VEGF expression. Such a complex treatment would be impractical in humans, but the prevention of injury and repair responses of the oxygen-injured rodent lung are proof of principal that VEGF dysregulation likely contributes to clinical BPD.

Other growth factors also have therapeutic benefits in animal models of BPD. Keratinocyte growth factor (KGF or FGF7) can protect newborn rats from death from pulmonary hypertension but not for the decreased septation in >95% oxygen over the first 2 weeks of life (115). Franco-Montoya et al. (116) noted that intraperitoneal KGF prevented neutrophil recruitment to the airspaces of >95% oxygen-exposed newborn rats with less decrease in DNA or cell proliferation, but the structural changes in the newborn lungs were not changed by KGF treatment. Thus, any treatment benefits of KGF in the rodent model were partial at best.

In contrast, hepatocyte growth factor (HGF) had more remarkable effects on newborn rodent lungs exposed to 90% oxygen. Intraperitoneal HGF partially corrected increased airway resistance and hyper-responsiveness, improved the alveolar simplification, and increased the microvasculature (117). Platelet-derived growth factor can have long-term effects on septation if its signaling is disrupted with a receptor antagonist even briefly (118). Therapy with PDGF has not been reported in BPD models. Subramaniam and colleagues (119) report that bombesin-like peptides predict the severity of BPD in ventilated preterm baboons, and an antibody to these peptides improved the septation. Clearly, multiple growth factors act in time and space to regulate lung development. Although animal models support growth factor–based treatments for BPD, caution is warranted as such therapies may have adverse effects on other developing systems.

Other Treatments for BPD in Animal Models

Glucocorticoid treatments have been used for the prevention of BPD (early treatments) and to treat the progression of BPD (120). Although there are concerns about the pleotropic developmental effects, dexamethasone can decrease the incidence of BPD and mortality. The presumed mechanism of action is as a potent anti-inflammatory. Cyclooxygenase-2 (Cox-2) is upregulated in the 60% oxygen exposure rodent model of BPD and in lungs with BPD (121). Therefore, Masood and colleagues (122) used a selective Cox-2 inhibitor to suppress prostaglandin production during oxygen exposure of newborn rats with remarkable results. The Cox-2 inhibitor prevented recruitment of neutrophils and macrophages and blocked the inhibition of alveolar septation and the increase in tissue fraction caused by oxygen. Choo-Wing et al. (121) used transgenic technology to overexpress interferon-γ and small interfering RNA to inhibit the endoplasmic reticulum stress pathway in hyperoxia-exposed newborn mice. A Cox-2 inhibitor or the siRNA prevented the arrest of alveolarization caused by INFα and hyperoxia. These results suggest inhibition of Cox should be an effective therapy for BPD, but indomethacin or ibuprofen treatments of infants to decrease intraventricular hemorrhage or to close the patent ductus arteriosus are not associated with a decrease in BPD (123). However, specific Cox-2 inhibitors have not been directly tested to prevent BPD.

TGF-β1 is an important mediator of both stimulation and inhibition of lung cell development and airway branching (124). Airspace fluids from infants with BPD contain increased amounts of TGF-β1 and antenatal intra-amniotic LPS greatly induce TGF-β1 in the lungs of fetal lambs (70). Nakanishi et al. (125) demonstrated the alveolar and vascular abnormalities caused by 95% oxygen in newborn mice can be partially prevented with a TGF-β neutralizing antibody.

Focal elastin bundles identify the sites of septation, and inhibition of elastin formation or abnormal distributions of increased amounts of elastin in saccular/alveolar walls inhibit septation. Ventilated and oxygen-exposed preterm lambs have inhibited alveolarization. Lung growth factors (VEGF, PDGF-A) and their receptors were decreased at 3 weeks (36). Elastin-related gene expression increased and discrete septation initiation sites were lost with prolonged ventilation of the preterm lamb. Remarkably, these effects on septation sites to uncouple the synthesis and assembly of elastin can occur within 24 h of mechanical ventilation in newborn mice (37). Using the ventilated newborn mouse model, Hilgendorff et al. (126) then showed that intratracheal treatment with the elastase inhibitor elafin prevented elastin degradation, TGF-β activation, apoptosis, and decreased the septation abnormality. This result was reproduced using newborn mice that overexpressed elafin (126). These experiments demonstrate a therapeutic strategy that simply decreases the elastin degradation that accompanies mechanical ventilation.

Other pathways activated by oxygen or bleomycin exposure of newborn rodents demonstrate both the complexity of the oxygen injury, but also how targeting single pathways can prevent much of the injury. Rho-kinase is a ubiquitously expressed protein that regulates numerous cell functions and proliferation. It is upregulated by bleomycin in the newborn rat, and inhibition of Rho-kinase decreases the BPD-like changes caused by bleomycin (127). B-catenin is another multifunctional signaling protein that is critical for normal lung development and remodeling and aberrant β-catenin signaling has been associated with BPD. In the 90% oxygen-exposed newborn rat model, an inhibitor of β-catenin mitigated the septation defect and pulmonary hypertension (128). A recent observation is that an extract from a Chinese traditional herb *Astragalus membranaceus* has anti-inflammatory effects and protects newborn rats from oxygen injury (129). Finally, infants with BPD often have hypercarbia. Although hypercarbia can

have deleterious effects on the lung (130), in the newborn rat hypercapnea blunted the influx of macrophages, and the resulting pulmonary hypertension caused by bleomycin (131). With use of TNF-α inhibitor, the benefits of hypercapnea were explained by fewer macrophages secreting TNF-α.

Ventilation Strategies to Decrease Lung Injury

High-Frequency Oscillatory Ventilation

Mechanical ventilation of the premature infant is a major contributor to lung injury that progresses to BPD. A strategy to minimize injury that was initially tested in preterm animals was high-frequency oscillatory ventilation (HFOV). Preterm baboons supported by HFOV required less supplemental oxygen and had fewer air leaks than animals supported with conventional mechanical ventilation (CMV) (132). All HFV animals survived for 11 days while 5 of 11 animals on CMV died within 48 h. In 6 h experiments, Jackson and colleagues (133) reported that premature *Macaca nemestrina* ventilated with HFV had better expanded lungs with less alveolar debris and better gas exchange. Other measures of lung injury were not different. A subsequent report with this primate model demonstrated that HFV when combined with surfactant treatment caused less injury than HFV or surfactant treatments alone (134). Yoder et al. (135) used very preterm baboons at 125 days gestational age (GA) who were exposed to antenatal steroids and treated with surfactant at birth to compare HFV with CV using tidal volumes of 4–6 ml/kg for 1–2 months. Prolonged HFV resulted in better lung mechanics, a lower oxygen requirement, and lower cytokine levels in airway samples at selected timesV when combined to 1 month of age. At autopsy, the HFV animals had better inflated lungs, but both groups had pathologic changes of BPD. Overall the animal studies support substantial benefits of HFV relative to CMV. Unfortunately, meta-analyses of 3,260 randomized infants found no net benefit of HFV for decreasing BPD (136). An individual patient analysis of nine trials to evaluate HFV relative to CMC also showed no BPD benefit for HFV (137). However, a recent report demonstrated better pulmonary function tests at 11–14 years of age for infants randomized to HFC relative to CMV (138). Overall, the animal testing was not predictive of clinical benefit.

Tidal Volumes and Continuous Positive Airway Pressure

The preterm lung can be easily injured with high tidal volumes as demonstrated by the effects of high tidal volumes following birth (31, 32). Animal models were used to evaluate chronic effects of tidal volumes and non-invasive ventilation strategies. Preterm surfactant-treated lambs were randomized to 20 breaths/min with 15 ml/kg tidal volumes or 60 breaths/min with 6 ml/kg tidal volumes for 3–4 weeks by Albertine et al. (139). Both ventilation groups had abnormal alveolarization relative to age matched non-ventilated controls. The high tidal volume animals had more impaired alveolarization and more elastin than the low tidal volume animals. Studies of similarly ventilated lambs showed decreased pulmonary microvasculature, interstitial edema and increased pulmonary arteriolar smooth muscle and elastin in both groups of animals (140).

Animal models also were used to test the hypothesis that non-invasive respiratory support would injure the preterm lung less than CMV. 125 days GA baboons were treated with surfactant and stabilized with CMV for 24 h before extubation to nasal CPAP for 4 weeks (141). As compared to fetal controls, the CPAP animals had enlarged thin walled air spaces, but with minimal injury compared to animals supported with CMV. A second study with preterm baboons evaluated an early 24 h extubation to CPAP or a delayed 5 days extubation to CPAP (142). The delayed CPAP group had worse oxygenation and more inflammation and higher proinflammatory cytokine levels in the airway samples than did the early CPAP group, but morphometry for alveolarization was similar between groups. Preterm lambs ventilated using either non-invasive high-frequency nasal ventilation or CMV with tidal volumes of 5–7 ml/kg were evaluated at 3 days for the effects of the ventilation style on mesenchymal cell apoptosis and proliferation (143). The non-invasive ventilation achieved more thinning of the mesenchyme with more apoptosis and less proliferation that supported alveolarization better than the CMV group. As with the HFO studies, these non-invasive ventilation approaches decreased a variety of indicators of lung injury better than CMV. However, comparisons of CPAP strategies with intubation, surfactant treatment, and CMV had only marginal benefits to prevent BPD in four trials in very low birth weight infants (144).

Preconditioning for BPD-Like Responses of Newborn Lungs

The animal models and treatments described so far have used a single lung injury – oxygen, bleomycin, or ventilation – to induce BPD-like changes in developing lungs. Clinical medicine is much more complex because of the multiple associations with preterm delivery. There is a large literature about how an antenatal exposure can modulate a postnatal exposure (18). A representative example is the report by Tang et al. (145) that demonstrates that fetal exposure of mice to intra-amniotic LPS causes a BPD phenotype in the newborn mice. Postnatal exposure to >95% oxygen increases the BPD-like abnormalities. However, postnatal exposure to 65% oxygen has the opposite effect of accelerated lung growth and attenuated pulmonary hypertension. A second example is the competing effects of intracervical *E. coli* and betamethasone on postnatal hyperoxic exposures in rats (146). The hyperoxic responses were increased by the antenatal inflammation but attenuated by the combination of inflammation and betamethasone. A post-delivery exposure such as hyperoxia also can aggravate lung injuries later in life. Newborn mice exposed to 100% oxygen for 4 days and allowed to recover in room air were much more sensitive to bleomycin-induced fibrosis or influenza virus as adults (147, 148). The important message is that one exposure that may cause a BPD phenotype may be substantially modified by a previous or subsequent exposure. These complex exposures can only be evaluated in animal models.

Summary

The animal models of BPD that use premature sheep, primates, or newborn rodents have injury responses that are similar to what is

known about the pathology of BPD. A cautionary note is that the pathology is only available for infants who have died of end stage BPD, with few exceptions. The range of abnormalities may be far greater for the majority of infants who survive BPD to remodel and grow their lungs. The interventions in animal models to decrease oxidant injury, inflammation, and volutrauma each show rather striking benefits for the major pathologies of decreased septation and microvascular development. Given the list of positive strategies enumerated in this review, effective treatments should be either in use or in late stages of development. But they are not.

My explanation is that the animals are studied as normal fetuses or newborns, and virtually all preterm infants are abnormal because of the abnormalities associated with very preterm delivery, primarily placental dysfunction, fetal exposure to infection, and fetal growth restriction (149). Further, >80% of the infants have been exposed to antenatal corticosteroids (1). Preconditioning events likely change each infant's responses to oxygen, ventilation, and inflammation. The clinical responses also are likely to be modulated by the genetic variability in humans that is greatly minimized in the animal models. For example, clinical interventions that have been extensively trialed such as antenatal corticosteroids and surfactant treatments required several hundred humans to be randomized to show clinically relevant effects such as decreased mortality (3, 4). Experiments with sheep or primates with group numbers of four to eight can show highly significant differences in physiological outcomes such as lung compliances or oxygenation. Effects of antenatal steroids or surfactant on BPD are minimal in human trials because of decreased mortality with more high-risk infants surviving.

However, the animal models of BPD have shaped our understanding of the disease. Care strategies have improved which has allowed the survival of smaller and more fragile infants. Less aggressive approaches to ventilation, better nutrition, and better control of postnatal infection are hard to document, but in my view are of substantial benefit to preterm infants. Finally, a major deficiency is the clinical definition of BPD that is based on a therapy – supplemental oxygen. The diagnosis does not directly inform the clinician about how the lungs are working or what the abnormalities may be. It may be most difficult to evaluate a therapy based on animal models if there is not more precision in knowing what components of BPD the therapy should improve. Therapies targeting specific pathways that are effective in animal models are most difficult to translate into a safe and effective therapy in preterm infants because of constraints on clinical research in this population. However, animal models will have a critical role in refining our understanding of the pathophysiology of lung injury and repair in the future. Research strategies should include more complex models with multiple and sequential fetal and/or newborn lung injuries. A focus on repair mechanisms may be more informative than studies of acute injury mechanisms. The identification of biomarkers that can be used to identify primary injury or repair pathways may help in the testing of treatments in infants, as surrogate indicators are preferable to long-term outcomes for studies in infants.

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Conflict of Interest Statement: I have consulted about therapies for RDS and BPD with Chiesi, Parma Italy and Abbvie, Chicago, USA. Chiesi has provided surfactant and Fisher & Paykel, Auckland, NZ, has provided supplies for studies of lung injury in preterm sheep performed in Australia. This research was supported by grants from the US National Institute of Health. There was no support for this work nor do I have patents or financial holdings that are related to this work.

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REVIEW
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Affect of early life oxygen exposure on proper lung development and response to respiratory viral infections

William Domm[1,2]*, *Ravi S. Misra*[1] and *Michael A. O'Reilly*[1,2]

[1] Department of Pediatrics, School of Medicine and Dentistry, The University of Rochester, Rochester, NY, USA, [2] Department of Environmental Medicine, School of Medicine and Dentistry, The University of Rochester, Rochester, NY, USA

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Greece

***Correspondence:**
William Domm,
Department of Pediatrics, School of Medicine and Dentistry, The University of Rochester, 601 Elmwood Avenue, Box 850, Rochester, NY 14642, USA
william_domm@urmc.rochester.edu

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Children born preterm often exhibit reduced lung function and increased severity of response to respiratory viruses, suggesting that premature birth has compromised proper development of the respiratory epithelium and innate immune defenses. Increasing evidence suggests that premature birth promotes aberrant lung development likely due to the neonatal oxygen transition occurring before pulmonary development has matured. Given that preterm infants are born at a point of time where their immune system is also still developing, early life oxygen exposure may also be disrupting proper development of innate immunity. Here, we review current literature in hopes of stimulating research that enhances understanding of how the oxygen environment at birth influences lung development and host defense. This knowledge may help identify those children at risk for disease and ideally culminate in the development of novel therapies that improve their health.

Keywords: hyperoxia, influenza A virus, innate immunity, lung development, prematurity

Introduction

Growing evidence suggest gene–environment interactions during critical stages of development profoundly influence health later in life. This concept of "developmental origins of health and disease," also called DOHaD, originated with a study by Dr. David Barker who showed that low birth weight correlated with increased risk of coronary heart disease in adults (1). DOHaD has now been linked to a wide variety of diseases in children and adults. Preterm birth, infection, tobacco smoke, and exposure to many inhaled pollutants can permanently impact lung development and immune function (2–4). Similarly, exposure to exogenous chemicals, malnutrition, and low birth weight correlates with poorer immune function (5–8). Even socioeconomic status and child abuse have been shown to influence a healthy lifestyle later in life (9). In 1983, the comedy movie *Trading Places* starring Dan Aykroyd and Eddie Murphy "tested" whether nature or nurture were responsible for distinguishing social hierarchy between two individuals. Although the question was never resolved in the movie, we are now beginning to appreciate 30 years later that gene–environment interactions influence children's health, in part, through metabolic and epigenetic reprograming of cells required for organ growth, regeneration, and immunity.

The human lung is designed to efficiently exchange oxidant gases between the environment and blood, and exclude or defend against inhaled pollutants that otherwise disrupts this process. When considering gene–environment interactions that influence lung function, the transition to

air at birth must surely be one of the most profound environmental changes that one will ever experience. In this singular moment, the delivery of oxygen and nutrients via the placenta is transferred, respectively, to the lung and gut. Both organs must therefore be developmentally mature and functional by this time. Proper development of the lung involves a complex set of transcription factors, morphogens, growth factors, and matrix molecules be expressed during precise developmental windows (10–13). Expression profiling studies have defined a pattern of gene expression wherein developmental genes are expressed first and genes involved in oxygen transport, protection against reactive oxygen species, and host defense are expressed near birth (14, 15). This "time-to-birth" program ensures that the lung is ready to breathe air and defend against environmental toxins at birth.

The interaction of genes with the oxygen environment at birth is disrupted when infants are born too soon. Many preterm infants develop bronchopulmonary dysplasia, a chronic form of lung disease characterized by alveolar simplification and restrictive airways (16). Mechanisms that promote BPD include genetics and maternal, fetal, or postnatal environments (17). It has been difficult to define which is most important for initiating or promoting disease, perhaps because BPD is clinically defined by the amount of oxygen used at a specific gestational age (18, 19). Fortunately, most preterm infants born >24 weeks gestation are surviving, albeit at the risk of developing a variety of lung and non-lung diseases later in life. Children born preterm often display reduced lung function, increased re-hospitalization following a respiratory viral infection, and incidence of non-atopic asthma (20, 21). They may also show neurodevelopmental delay and have greater risk for high blood pressure and heart disease as adults (22, 23). The annual cost of treating children in the United States who were born prematurely in 2005 was $26.2 billion dollars, of which 10% was just for treating infants with BPD (http://www.nhlbi.nih.gov/new/press/06-07-26.htm). Hence, there is an urgent need to understand how premature birth is a developmental antecedent of poorer health later in life.

The pathogenesis of BPD and the health sequela of survivors is a complex and poorly understood process, perhaps because it is a multi-organ disease originating from abnormal gene–environment interactions. Recognizing that there is a genetic program designed to create the lung and afford it anti-oxidant and innate immune defenses by birth, it seems rather obvious that preterm birth will disrupt the timing of when specific genetic programs need to be completed or in place to properly allow the lung to transition to an oxygen-rich environment. Therefore, identifying genetic variants that predispose to preterm birth may also identify variants that correlate with BPD. A screen of single-nucleotide polymorphisms identified two genes (CRHR1 and CYP2E1) acting in the fetus and four genes (ENPP1, IGFBP3, DHCR7, and TRAF2) in the mother that predisposes to preterm birth (24). But, interestingly none of these genes have been detected in other studies seeking to find variants that predispose preterm infants to BPD (25, 26). In fact, the few weak candidates detected in one study were not detected in another, suggesting that BPD is not entirely a genetic disorder. On the other hand, widespread methylation was detected in the blood of extremely preterm infants, suggesting that there were changes in blood cell development, composition, and perhaps immune function (27). Since these changes in methylation resolved by 18 years of age, they may not be responsible for the long-term health effects reported in people born preterm. Therefore, genetic susceptibility to BPD is more likely to represent genetic variants that modify how cells respond to an environmental stress, such as infection or the transition to air too soon.

Environmental stresses known to promote BPD include prenatal and postnatal infections, and oxygen or ventilator-induced damage to the lung. In both cases, inflammation and oxidative stress or damage to the developing lung seems to be a primary driver of BPD. Preterm infants are deficient in anti-oxidant enzymes and are therefore susceptible to oxidative stress, whether initiated by inflammation or supplemental oxygen therapies in the preterm infant (28, 29). Lungs of preterm infants are often underdeveloped and cannot adequately exchange oxygen and carbon dioxide. Supplemental oxygen supported by ventilation is often used to improve blood oxygen levels and prevent hypoxemia. However, it is now clear that high levels of oxygen can disrupt development of the lung and is a risk factor for neurodevelopmental delay, retinopathy, and probably other diseases attributed to preterm birth (30). Oxygen-induced damage can also elicit an inflammatory response, subsequently compounding the oxidative stress to the lung. Consistent with oxygen playing a role in the pathogenesis of BPD and the long-term respiratory complications associated with preterm birth, anti-oxidant therapies have proven partially effective in alleviating lung disease in humans and in animals exposed to high oxygen (31–34). Because the pathogenesis of neonatal oxygen exposure in humans and in animal models has been recently reviewed (19, 35–40), the following discusses oxygen-induced changes in lung development in relationship to how it also perturbs host response to respiratory viral infections.

Proper Lung Development

The pulmonary system, in highly simplistic form, can be described as the co-branching of air conducting and blood circulating systems that, due to simultaneous and congruent branching, efficiently interact for proper gas-exchange and subsequent systemic circulation of oxygen. In humans, gas-exchange is accomplished by diffusion through squamous epithelial cells in the alveolar saccules of the mature lung. Branching morphogenesis of the airways that concludes with formation of the alveolus leads to an impressive pulmonary surface area of around 70 square meters with a thickness of 0.1 um capable of supporting an oxygen consumption of 250–5500 ml/min (41, 42). This developmental program progresses through five successive stages. The mammalian lung undergoes five stages of maturation that begin with the embryonic stage, followed by the pseudoglandular, canalicular, saccular, and ending with the alveolar stage (**Figure 1**). The timing of these stages during fetal and postnatal periods varies between species, including between humans and mice. This is important when attempting to model human diseases in experimental animals. For example, many preterm infants born today are in the saccular phase of lung development, which pathologically corresponds to e17.5 to postnatal day 4 in mice. Hence, the mouse

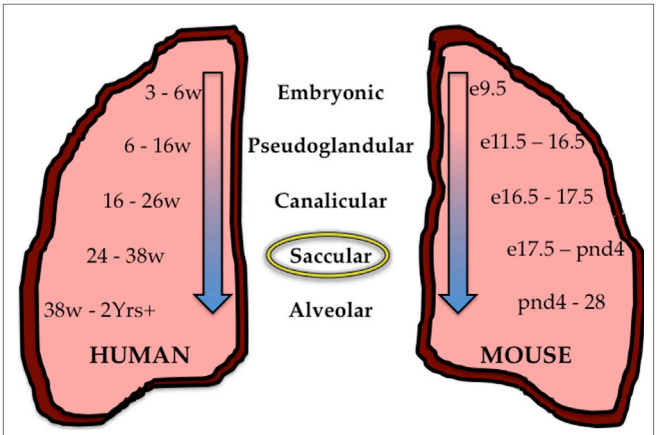

FIGURE 1 | Stages of lung development in the human and mouse. During development, the human (mouse) lung undergoes five successive stages of development; The Embryonic stage 3–6 weeks (e9.5–11.5), the Pseudoglandular stage 6–16 weeks (e11.5–16.5), the Canalicular stage 12–26 weeks (e16.5–17.5), the Saccular stage 24–38 weeks (e17.5–PND4), and the Alveolar stage 38 weeks–2+ years (PND4–28). Preterm children who survive are often born between 24 and 38 weeks of age and are in the saccular stage of development (circled) corresponding to the saccular stage in the mouse from e17.5–PND4.

is an appropriate experimental model for studying how too much oxygen can perturb saccular development in preterm humans. Additional details on factors controlling lung development have been reviewed elsewhere (10, 12, 41, 43).

Successive developmental stages are defined by changes in lung morphology. In the embryonic stage, the pulmonary branching pattern originates and two distinct lobes are formed. The pseudoglandular stage marks the appearance of numerous terminal buds projecting away from the initial two lung lobes and recent work has defined the patterns as domain branching, planar, and orthogonal bifurcation budding (44). During the canalicular stage, epithelial tubules form with large terminal buds while the mesenchyme separates into dense subsets between future alveolar septa. Specialized epithelial cell types and alveolar sacs emerge during the saccular stage of development. Squamous type I epithelial cells form the lining of the alveolar sacs with cuboidal type II epithelial cells interspersed. Thinning of the mesenchyme along with an increase in extracellular matrix allows for expansion of these alveolar sacs culminating in the alveolar stage where dense connective tissue, containing cartilage and smooth muscle, surrounds the airways. The timing of developmental completion, leading to the formation of alveolar sacs, varies between species. In mice and rats, alveolar development concludes mainly postnatally characterized by lung expansion and alveoli subdividing into smaller gas-exchanging units (45). Importantly, this morphogenic process has been accompanied by blood vessel morphogenesis that concludes with capillary networks residing in close proximity to the alveolar epithelium.

It is often written that the normal adult mammalian lung contains approximately 40 different cell types, yet the origin of this statement seems to have disappeared in the historical literature. However, it should not be surprising to find that this is a gross underestimation when one considers how expression of cell surface receptors has markedly increased the diversity of leukocytes present in the lung (46). The emerging use of microfluidic single-cell RNA sequencing is also uncovering an equally rich diversity among non-hematopoietic cell populations (47, 48). Pulse-chase labeling with H-thymidine, cell-restricted fluorescent reporter genes, and cell-specific ablation with toxins has identified region-specific niches containing stem cells required for proper lung development and repair (49). Unique specific stem cell niches may therefore have evolved to facilitate repair of specific areas of the lung damaged by region-specific toxins. Since perinatal exposures influence saccular and alveolar phases of development, the following briefly focuses on progenitor cells controlling distal airway and alveolar development and regeneration.

The region where the airway meets the alveolus has been termed the bronchoalveolar duct junction (BADJ) (50). The distal airway epithelium contains Clara (now called Club) cells defined by their cuboidal appearance and expression of secretoglobin family 1A, member 1 (Scgb1a1), also called Clara Cell Secretory Protein (CCSP) or uteroglobin. During recovery from naphthalene depletion, a population of Club cells proliferates from neuroendocrine bodies and from the BADJ (51, 52). These bronchoalveolar stem cells (BASC) express airway Scgb1a1, alveolar Type II surfactant protein (SP)-C, the stem cell markers Sca-1, and CD34, but not CD45 (53). These BASCs are able to self-renew and maintain expression of both airway Scgb1a1and alveolar SP-C expression when cultured on irradiated mouse embryonic fibroblasts. However, their importance in defining airway and alveolar epithelial cell development and repair remains unclear because they proliferate less frequently than Type II cells in a post-pneumonectomy model of lung regeneration (54).

A label-retaining population of airway cells expressing Scgb1a1 and the stem cell markers Oct-4, Sca-1, and SSEA-1 has also been identified in BADJ (55). These cells can be maintained *ex vivo* for several weeks, but have the capacity to express SP-C and T1α when cultured on Type I collagen. Fate-mapping studies using Scgb1a1-driven reverse transcriptional transactivator (rtTA) gene or Cre fused to an estrogen responsive binding site (CreER) gene to durably label Scgb1a1+ cells with LacZ or fluorescent proteins has provided new insight into the ability of airway Scgb1a1+ progenitors to repopulate alveolar cells. Depending upon the model and the timing of activation, airway Scgb1a1+ progenitors contribute to ~10–50% of adult type II cells during normal postnatal lung development (56–60). These cells also contribute to alveolar repair when adult mice are infected with Influenza A Virus (IAV) or injured with bleomycin, both of which damage alveolar type II cells (59). Interestingly, they do not participate in repair when mice are exposed to hyperoxia or naphthalene (58). Since hyperoxia injures alveolar type I cells, and naphthalene injures airway Club cells, these two studies suggest Scgb1a1 + cells may serve as precursors for themselves and type II cells.

Analogous to studies using naphthalene to ablate airway Clara cells, exposure of adult mice, rats, or monkeys to oxidant gases (hyperoxia, ozone, or nitrogen dioxide) kills alveolar type I epithelial cells (61–63). Pulse-chase labeling studies with H-thymidine indicate type II epithelial cells proliferate and differentiate into type I cells following injury (64–66). Emerging evidence suggests

that subpopulations of type II cells exist and T1α, a protein expressed by Type I cells, has been shown to co-localize with the Type II cell-specific lectin Maclura pomifera (67). Tri-transgenic mice containing the rat airway CCSP promoter driving rtTA, the otet-Cre gene, and the LacZ/EGFP (Z/EG) reporter identified a lineage of epithelial cells that defines airway Club and a small population of alveolar Type II cells (68). Recently, single-cell RNA sequencing revealed the existence of four distinct populations of type II cells (48). Alveolar type I cells have historically be thought to be the most terminally differentiated cell of the lung whose sole function was to facilitate gas-exchange and maintain barrier function (64–66). However, a study showing that type I cells isolated from rats can proliferate *ex vivo*, express the stem cell protein Oct-4, and can be induced to express SP-C and Scgb1a1 has challenged this conclusion (69).

Pulmonary Response to Influenza A Infection

As the lung evolved to efficiently exchange oxygen and carbon dioxide, so did an innate immune system comprised of specialized epithelial resident cells and circulating immune cells that function to recognize and clear a variety of inhaled pathogens and toxicants. Failure to detoxify the airspace can result in significant disease and even death. These defenses are most likely designed to respond to inhaled pathogens, like respiratory viruses, which were present in the environment before vertebrates migrated onto land. We therefore will discuss the current understanding of the pulmonary interactions with respiratory infections, primarily focusing on IAV, in an attempt to build a greater understanding of the poor response experienced by children born prematurely.

Viral respiratory infections have been found to afflict preterm infants at a higher rate than full term controls. Respiratory Syncytial Virus (RSV), human Rhinovirus (RV), and Bocavirus infection of children less than 14 years of age hospitalized over a 7-year study period were described (70). The authors found that children who were preterm exhibited a higher rate of infection with human metapneumovirus and parainfluenza virus as compared to controls (70). Additionally, a recent study describes extremely and moderately preterm infants facing a 3.6 times increased risk of being hospitalized due to respiratory infection, likely from RSV or RV, in the first year of life (71). Preterm infants hospitalized due to RSV were found more likely to wheeze in the first six years of life and experience decreased quality of life versus those infants who were not hospitalized due to RSV infection (72). RV infection of preterm infants also increases the risk of developing wheeze and requiring respiratory medicines in the first year of life, and can be the source of serious lower respiratory tract infections (73–76). A recent NHLBI workshop report recommends identifying prophylactic approaches to prevent RSV and RV infections to help lessen the burden of asthma development in childhood (77), however determining when the use of such prophylaxis is complicated (78). Thus, infants born preterm face serious consequences in response to respiratory viral infections.

In human pediatric populations, RSV is more common in infancy (first two years of life) while IAV is generally more common in school age children (79, 80). Gaining a better understanding of how early life oxygen exposure affects responses to respiratory viral infections necessitates the use of animal models. While different species have shown utilization in RSV modeling, each has advantages and disadvantages (81, 82). Human RSV does not efficiently replicate and leads to non-significant disease and mortality in mouse models, making it difficult to model how it is perturbed in preterm children (82). This is in contrast to IAV mouse models that have proven robust viral replication and disease that closely model human disease. Here, neonatal oxygen exposures that have been shown to promote BPD-like lung disease in mice have also been shown to alter the response to IAV infection (35). Understanding how the oxygen environment at birth disrupts the host response to IAV may provide insight into how it influences the response to RSV and other respiratory viruses.

IAV annually causes global seasonal epidemics but also novel IAV occasionally arise leading to global pandemics. The most notorious of which was the pandemic of 1918 and the most recent the 2009 swine-flu pandemic (83). Significant insight into IAV–host interactions has historically occurred through *in vitro* investigations. A much greater understanding of this virus–host interaction, prior to, during, and following significant pathological outcomes *in vivo*, has been hampered due to a lack of traceable reporter expressing IAV that retain full virulence as well as other technical problems. Recently, IAV–host interactions and *in vivo* dynamics following infection have been investigated utilizing reporter expressing recombinant IAV (84, 85).

The first step in IAV infection involves the recognition of sialic-acid (SA) moieties on the surface of susceptible cells by the viral hemagglutinin (HA) protein. Human IAV primarily infect via α2-6 SA residues and avian IAV by α2-3 linked residues. In healthy humans, α2-6 SA has been primarily found on the epithelial (ciliated and non-ciliated) and goblet cells of the upper respiratory tract in humans (86). Avian like α2-3 SA has primarily been found on non-ciliated bronchiolar and alveolar type II cells in the lower respiratory tract (86, 87). Viral attachment and histochemical studies have revealed human IAV primarily interacting with the upper respiratory tract through ciliated epithelial cells, goblet cells, as well as to type I alveolar epithelial cells, to varying extents (86–89). Contrasting with human IAV, avian IAV has been shown to primarily attach to alveolar epithelial type II cells, alveolar macrophages, and bronchiolar non-ciliated epithelial cells (89, 90). Sialic-acid receptor expression is a good correlate of IAV binding based upon histochemical studies. Although human IAV is of primary concern for understanding infection of the population discussed in this review, understanding avian IAV infection is imperative in the face of novel viruses entering the human population.

Both human and avian IAV can infect human airway epithelial cultures with human IAV preferentially target non-ciliated airway cells whereas avian IAV infect ciliated populations (91, 92). Alveolar type II cells have also been demonstrated as a site of IAV infection and replication although their importance to human disease is currently unclear. Human alveolar type II cells were infected by IAV in a primary cell culture system (93). Alveolar type II cells are imperative for the maintenance of the alveoli by

producing and secreting surfactant as well as being a renewable source for themselves and type I alveolar cells. Although poorly understood, the affect of IAV infection of type II cells has been shown to affect their phenotype and subsequent innate immune responses (93). Taken together, IAV tropism as it relates to human disease requires further investigation. Differences based on the strain of IAV used and type of assay utilized must be clarified for a greater understanding of human disease.

The source of cells responsible for pulmonary regeneration following viral injury is currently an active area of research. Bronchiolar epithelial cells expressing p63 were found to rapidly expand and disseminate to areas of lung injury following IAV infection and repair (94). This cell population was also found to have the ability to form "pods" in both bronchiolar as well as alveolar regions following injury caused by IAV. Keratin 5 expression (Krt5) was also shown to map to these regions and, importantly, was only detected following IAV infection, during reparative processes (94). These p63/Krt5 + cell populations therefore may act as distal airway stem cells and serve as the source for alveoli cell regeneration following injury and recently these p63/Krt5 + cells were found to recapitulate alveoli following epithelial injury by IAV (95). This unique population also has the ability to form alveoli-like structures when delivered to IAV-infected lungs minimizing virus-induced pathology (95).

Oxygen Perturbation of Proper Lung Development and Innate Immunity

As discussed previously, the transition to an oxygen environment at birth may be one of the most profound environmental changes one will ever experience and can lead to disease when it occurs inappropriately. Lungs of infants born preterm are often in the saccular phase of development. Alveolar regions at this time have yet to develop into true gas-exchanging structures, which is why many preterm infants develop respiratory distress. Furthermore, the capillary network surrounding the alveolus, which shuttles oxygen to the circulation, has yet to effectively complement the alveolus (96). Despite the life-saving efficacy of supplemental oxygen treatment during this critical time, growing evidence suggests that this treatment contributes to bronchopulmonary dysplasia (BPD), a chronic lung disease that is characterized by alveolar simplification and restrictive airways (16, 30). Oxygen-dependent changes in genes specifying lung structure and cell phenotype are likely to impact cells and molecules involved in innate immunity required for a proper host response to respiratory viral infection (**Figure 2**). This includes alveolar epithelial type II cells, goblet cells, eosinophils, macrophages, dendritic cells, T cells, B cells, and innate lymphoid cells, in addition to soluble mediators produced by these cells, including SPs, cytokines, chemokines, and mucus proteins mediate innate immunity (97–100). In other words, early life oxygen exposure or other oxidative stresses may drive the development of long-term lung disease by disrupting a delicate balance of cell communication between genes controlling lung development and innate immunity.

One hallmark of supplemental oxygen treatment at birth is the development of a highly simplified alveolar epithelium. Although incompletely understood, this may develop due to oxidative stress

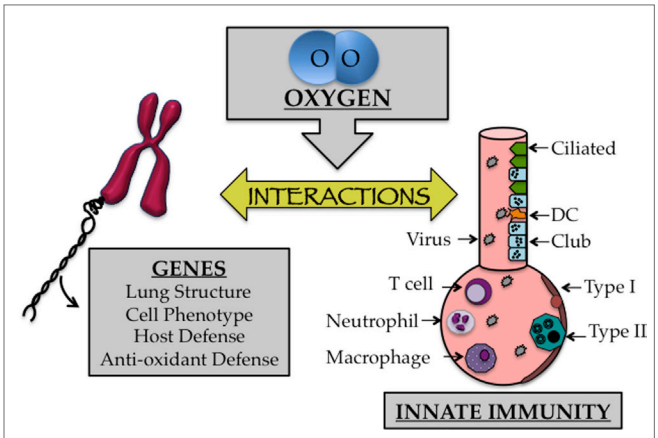

FIGURE 2 | The early life oxygen environment affects changes in genetic as well as innate immune mechanisms. Cartoon depicting the affect early life oxygen environment imparts on genes that specify lung structure and function with cells involved in innate immunity.

or an aberrant immune response that suppresses angiogenic factors (101). In mice, alveolar epithelial type II cells expand rapidly following neonatal hyperoxia compared to room air control littermates (102, 103). Following recovery in room air however, this population is significantly pruned (102, 103). This results in a significant decrease in the pool of alveolar type II cells later in life. Concomitant with the loss of type II cells, markers for type I alveolar epithelial cells increase during the same time frame. Currently, the source of these cells is unclear; however, evidence suggests that type II alveolar cells lost during recovery in room air are not the source of these cells (102). Further fate-mapping studies of type II and type I cells during and following exposure to hyperoxia should help to clarify the intricate balance and source of these cells. Regardless, the loss of type II cells may adversely impact alveolar repair as well as the production of innate immunity. Indeed, adult mice exposed to hyperoxia exhibit persistent and altered immune responses, fibrosis (**Figure 3**), and increased mortality compared to room air littermates when infected with a sublethal dose of IAV (32, 104, 105). The altered host response was not attributable to CD8 T cells and therefore the pathology is not likely due to a defect in viral clearance (106). While reduced numbers of type II cells did not negatively impact surfactant pools (107), it reduced expression of the antiviral protein eosinophil-associated RNase 1 (Ear1) detected in some type II cells (104). Reduced expression of Ear1, while conceptually attractive, does not solely account for the fibrotic phenotype observed in IAV-infected mice that have been previously exposed to hyperoxia as neonates. This is because neonatal hyperoxia has also been shown to enhance the severity of fibrosis in the neonatal hyperoxia model following bleomycin administration (108). Hypothetically, the loss of some type II cells may impact the orderly innate immune response releasing cytokines, chemokines, and SPs that are the first responders following IAV infection (109).

One example is monocyte chemoattractant protein-1 (MCP-1), which has been found to be selectively increased following IAV infection in a model of neonatal hyperoxia (105, 110). MCP-1 plays important roles in the recruitment of monocytes, T cells,

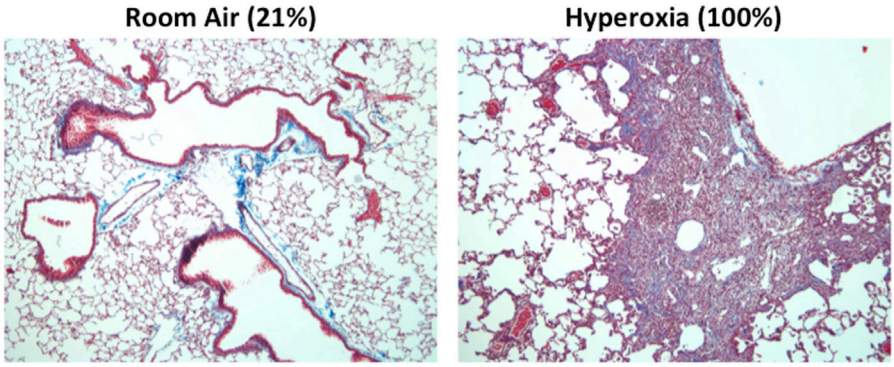

FIGURE 3 | Mice exposed to hyperoxia at birth develop fibrosis after influenza A infection. Adult (8-week old) C57Bl/6J mice exposed to room air (21% oxygen) or hyperoxia (100% oxygen) between postnatal days 0–4 were infected with 120 HAU of influenza A virus (H3N2). Trichrome staining revealed extensive collagen deposition and inflammation in infected mice exposed to neonatal hyperoxia 14 days post infection. This pathology was not evidence in infected siblings exposed to room air at birth.

and NK cells to sites of infection and has been shown to protect against viral and bacterial challenges (111, 112). However, aberrant MCP-1 control has also been associated with lung disease in children and adults (113, 114). While MCP-1 is an attractive target, it has recently been shown that MCP-1 is not solely responsible for the enhanced respiratory sequelae observed following IAV infection in neonatal hyperoxia-treated mice (105). This suggests that increased MCP-1 production may be an effect rather than a driver of the mechanisms leading to enhanced respiratory disease due to neonatal oxygen exposure.

In addition to an imbalance in alveolar type II cells, there are many other pulmonary innate immune mechanisms that might be affected by oxygen at birth. Animal models have identified several innate immune factors common in BPD-like lung injury. These include alterations in IL-6, IL-8, TNF-α, TGF-β, macrophage inflammatory factor-1α, IL-1β, MCP-1 MCP-2, CXCL-1, and CXCL-2 (115). Recent work also has identified mast cells as being present in the lungs of pediatric subjects who were diagnosed with BPD prior to death (100). Members of the IL-6 cytokine family have been shown to have fibrotic potential, which could contribute to lung disease (116). The compliment subunit C5a plays a role in neutrophil recruitment to the mouse lung following IAV infection and may be a potent inducer of hyperoxia-mediated lung injury via recruitment of macrophages, neutrophils, and lymphocytes, and increased expression of IL-6, TNF-α, and MCP-1 occurs (117, 118). Furthermore, C5a has been shown to increase TGF-β1 in primary human small airway epithelial cells, which could then contribute to the development of fibrosis (119). Thus, multiple factors could lead to the accumulation of C5a, which could induce inflammation in the lungs of preterm infants. Some of these factors have been proposed targets to prevent the development or to treat patients with BPD (120).

Several recent studies illustrated effects that hyperoxic stress imparts on the innate immune system. For instance, macrophages exposed to hyperoxic conditions experience cell cycle arrest and showed impaired phagocytic and chemotactic activity (121, 122). GM-CSF is critical for the maintenance of alveolar macrophages and hyperoxic stress has been demonstrated to decrease levels of GM-CSF via destabilization of mRNA in primary AEC cell cultures (123). Other studies indicate that the decrease in GM-CSF mRNA is due to upregulation of the microRNA molecule, miRNA 33 (124). This same publication illustrates the complex nature of hyperoxia by demonstrating that T cells actually up-regulate GM-CSF in response to hyperoxic stress (124). Taken together, this highlights the critical importance of macrophage balance on phenotype.

Macrophages have been shown to play a role in the development of alveoli (125). If these cells become more inflammatory in nature, such as experienced due to hyperoxic stress, they likely will contribute to lung pathology (126). A recent study illustrates that overexpression of TGF-β1 in the lung leads to the accumulation of inflammatory macrophages in a TGFβR2-dependent manner (127). Given the role for alveolar macrophages in activating T cells, it is possible that regulatory function of CD4 T cells could be compromised by pro-inflammatory macrophages found in the lung (128). In fact, active research is being conducted to try and target inflammatory macrophages to treat lung disease (129).

Neutrophils play a prominent role in the pathology of many lung diseases, including BPD (130). In a mouse model of hyperoxia, histological damage is preceded by neutrophil infiltration into the lung following a wave of macrophage recruitment (131). Several studies using animal models of hyperoxia show that reducing neutrophil infiltration correlates with decreased lung disease (132–134). Neutrophils play a complex role in the mechanism of inflammatory disease and it has recently been suggested that neutrophils can play an anti-inflammatory role in addition to their common pro-inflammatory role (135).

Human studies have reported an unexpected alteration in neutrophil counts in preterm infants, which could relate to the risk of preterm infants developing lung disease (136–138). Of note, one study reports that infants with respiratory distress syndrome born less than 32 weeks gestational age who develop BPD have elevated levels of IL-6 and IL-8 in tracheal aspirates prior to the influx of neutrophils versus those who do not develop BPD (139). A decrease in CD18 and CD62L on circulating neutrophils in the first 4 weeks of life in preterm infants was associated with

the development of BPD (140). Additionally, increased serum levels of neutrophil-associated gelatinase-associated lipocalin in preterm infants born less than 31 weeks of gestation was predictive for the development of BPD (141). Of note, children who were born less than 32 weeks gestational age have higher IL-8 and neutrophil cell counts in sputum at the preschool age, which illustrates long-term consequences in lung inflammation due to preterm birth (142). Thus, more studies are needed to understand how hyperoxia could alter the function of neonatal neutrophil function, which could then affect the development of inflammatory lung disease later in life.

It is becoming more apparent that respiratory disease pathology varies greatly and that unique subtypes of disease exist. Many of these subtypes display unique alterations in the skewing of the immune system toward a Th1, Th2, or Th17 response (143). The endotype of disease tends to track with the type of T cell skewing with a Th17/neutrophilic response being more damaging than other types of disease, and this is intimately related to the stimulatory conditions of activated T cells (144). In a study of extremely preterm infants (born <32 weeks GA) RV infection was shown to induce a Th2 and Th17 response, and IL-4 production was related to severity respiratory morbidity (145). Furthermore, alterations in T regulatory cells have been described in humans with respiratory disease (146). An important consideration is that T regulatory cells are associated with inhibition of fibroblast proliferation and in vascular repair in the lung following injury (144). Given the surprising finding that cord blood contains T cells with an activated/memory phenotype, it is possible that these cells are poised to contribute to inflammatory lung disease (147). Recent work has also reported decreased CD4 T cells in cord blood from preterm infants who develop moderate BPD (148). Despite the finding that cytotoxic T cell function is not altered in mice exposed to hyperoxia followed by IAV infection (106), it is possible that CD4 T cells play a role in hyperoxia-mediated lung damage in humans and in the development of disease later in life. However, small animal models of oxygen effects on BPD do not support this hypothesis.

In adults, oxidative stress plays a role in COPD disease progression (149). It is possible that changes in the oxidative state of the lung due to chronic oxygen exposure in preterm infants could change how cells from the immune system respond to environmental exposures by altering cellular function or the types of cytokines that are produced (150–153). These cytokines could work in concert with cell types in the lung, including epithelial cells and innate lymphoid cells, known to produce pro-inflammatory and pro-fibrotic factors under certain conditions (154). One recent report demonstrates that reactive oxygen species in the lung can alter signaling of the inflammasome, leading to increased inflammation (155). One cell lineage receiving a great deal of attention is the innate lymphoid cell, which is a bone-marrow derived population found at mucosal surfaces, including the lung. They have the ability to generate high levels of cytokines that can influence the balance of the immune system (156). Much like cells in the adaptive immune system, they can be skewed to express transcription factors and produce cytokines consistent with Th1, Th2, and Th17 CD4 T cell lineages and play an essential role in responding to infection (157, 158). Of particular interest, ILC2 cells have been shown to play a role in the pathogenesis of lung disease by contributing to a Th2 T cell response (159, 160). IL-13 is a Th2 cytokine that, when overexpressed in the lung, results in oxidative damage to peripheral blood cells (161). Of note is that oxidized guanidine perpetuates the inflammatory response (162). A related inflammatory mechanism could be present with complexes of oxidized high-mobility group box protein 1, which has been shown to induce hyperoxia-mediated lung inflammation (136, 163). It is tempting to speculate that exposure to hyperoxia could contribute to this inflammatory loop of chronic lung disease through the induction of oxidized DNA.

Taken together, the balance of redox state within the lung is of critical importance in preventing chronic lung disease. It is very likely that early life exposure to hyperoxia changes this balance, which could result in permanent lung injury. Alterations in function of immune cells, including but not limited to CD4 T cells, neutrophils, and macrophages, likely play a major role in this development of lung disease. Importantly, pulmonary cells that produce innate immune molecules, like type II epithelial cells, might also be depleted or epigenetically modified in their ability to respond to injury (102). Taken together, it is likely that low levels of inflammation are present following exposure to hyperoxia, which could perpetually contribute to lung disease.

A Perspective on Oxygen as a Goldilock's Modifier of Respiratory Health

If we accept that high levels of oxygen at birth can alter children's health, does low levels of oxygen at birth also affect children's health? Indeed, there is growing evidence that gene–environment interactions influences health of people living at high altitude (low oxygen). Populations of Tibetans, Ethiopians, and Andeans living at >2.5 miles or between 11 and 13% oxygen exhibit resistance to hypoxemia, and develop larger lungs and hearts. These phenotypic changes appear to be genetically fixed in Tibetans and Ethiopians, but not in Andeans. Between 2010 and 2014, single-cell gene analysis and whole-exome sequencing identified haplotypes in the prolyl hydroxylase EGLN1, hypoxia-inducible factor (HIF)-2α, and peroxisome proliferator-activated receptor (PPAR)-α genes that correlated with lower hemoglobin levels in Tibetans (164–167). These haplotypes are not detected in Ethiopians. Instead, haplotype changes in the retinoic acid orphan receptor have been detected, which is interesting because this receptor dimerizes with HIF-2α (168). Taken together, this suggests that Tibetan and Ethiopian populations adapted separately to hypoxia through a common EGLN-HIF signaling pathway. Genetic changes conferring resistance to hypoxia have yet to be detected in Andeans and the hypoxic-resistant phenotype is only present in children born at high altitude (169). This implies Andeans acclimatize to an environmentally low level of oxygen at birth.

Regardless of how adaptation at high altitude is achieved, it maladaptively influences long-term health. When compared to people living at sea level, high-altitude natives have increased risk for cardiovascular disease particularly related to cardiac hypertrophy (169). A zip code study of children born at high altitude in Colorado suggests that birth at high altitude increases

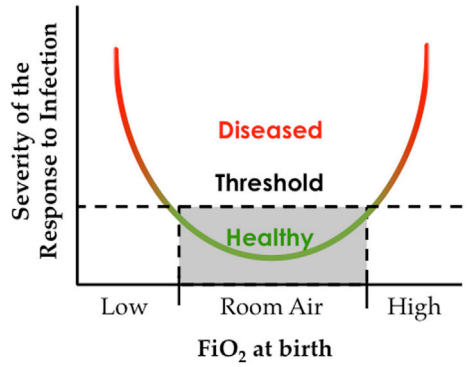

FIGURE 4 | The oxygen environment at birth affects the severity of respiratory viral infection later in life. Hypothetical graph depicting how exposure to low or high inspired oxygen at birth can increase respiratory morbidity following a respiratory viral infection.

re-hospitalization following infection with RSV (170). Living at high altitude may also reduce brain activity (171). High-altitude natives may have lower rates of obesity (172), but are often born small for gestational age and exhibit transient growth delay with compensatory catch-up growth (169, 173). Some of these health risks may mirror those seen in children who had sleep apnea, placental insufficiency, or cyanotic congenital heart disease as infants. Hence, adapting to low oxygen at birth causes similar maladaptive changes to children's health as high oxygen exposure.

This Goldilocks effect of oxygen reflects the convergence of an oxygen environment on genes present at birth, some of which have fixated changes that maintain the response to hypoxia even at sea level. Genetic changes that influence the response to high oxygen used to treat preterm infants have yet to be identified, perhaps because there is no evolutionary pressure or memory for adapting to hyperoxia. However, recognizing that the response to oxygen is non-linear, studying adaptation to low oxygen may help us understand adaptation to high oxygen (**Figure 4**).

In the preceding sections, we have highlighted the current understanding of normal pulmonary development and how it is perturbed due to premature birth. These changes become exasperated due to neonatal oxygen exposure that affects the pulmonary epithelium, angiogenesis, and the innate immune system in the developing infant. Great strides have recently been realized in both the treatment and understanding the mechanisms leading to sequelae later in life in this susceptible population. Our hope is that this review has left the reader with an appreciation for previous work as well as highlighting future areas of research that are warranted. These include but are not limited to gaining a more complete understanding of the molecular programing that drives development and regeneration of the respiratory epithelium that will allow for a better appreciation of the affects an immature lung experiences due to premature birth into an oxygen rich environment. Infants born prematurely, and likely provided oxygen, experience enhanced disease due to respiratory infections later in life. Understanding what pulmonary cell types are principally infected by various respiratory pathogens, like IAV, in healthy subjects precludes our understanding of the cell-specific alterations occurring in preterm infants later in life. Although cell-specific pulmonary tropism of IAV is unlikely to drastically change in this population, it may prove that a cell-specific imbalance in these aberrant lungs drives enhanced disease. It is also clear that genes involved in directing lung development overlap with those of the pulmonary innate immune system (97). It is therefore likely that overall respiratory health is accomplished by an interaction with oxygen at birth that influences the developmental trajectory of the lung and pulmonary innate immune system. A better understanding of how the oxygen environment at birth influences gene–innate immune interactions could help identify children at risk for disease and ideally treatments that improve their health.

Author Contributions

The design, writing, and editing of this manuscript was done with equal participation and intellectual contributions by WD, RM, and MO. Final editing and manuscript preparation was performed by WD.

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REVIEW
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Understanding the Impact of Infection, Inflammation, and Their Persistence in the Pathogenesis of Bronchopulmonary Dysplasia

*Jherna Balany and Vineet Bhandari**

Section of Neonatology, Department of Pediatrics, St. Christopher's Hospital for Children, Drexel University College of Medicine, Philadelphia, PA, USA

The concerted interaction of genetic and environmental factors acts on the preterm human immature lung with inflammation being the common denominator leading to the multifactorial origin of the most common chronic lung disease in infants – bronchopulmonary dysplasia (BPD). Adverse perinatal exposure to infection/inflammation with added insults like invasive mecha nical ventilation, exposure to hyperoxia, and sepsis causes persistent immune dysregulation. In this review article, we have attempted to analyze and consolidate current knowledge about the role played by persistent prenatal and postnatal inflammation in the pathogenesis of BPD. While some parameters of the early inflammatory response (neutrophils, cytokines, etc.) may not be detectable after days to weeks of exposure to noxious stimuli, they have already initiated the signaling pathways of the inflammatory process/immune cascade and have affected permanent defects structurally and functionally in the BPD lungs. Hence, translational research aimed at prevention/amelioration of BPD needs to focus on dampening the inflammatory response at an early stage to prevent the cascade of events leading to lung injury with impaired healing resulting in the pathologic pulmonary phenotype of alveolar simplification and dysregulated vascularization characteristic of BPD.

Keywords: premature newborn, chronic lung disease, cytokines, sepsis, hyperoxia, mechanical ventilation

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***Correspondence:**
Vineet Bhandari
vineet.bhandari@drexelmed.edu

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INTRODUCTION

Bronchopulmonary dysplasia (BPD) is the most common chronic respiratory disease affecting infants wherein the developmental program of the lung is altered secondary to preterm birth of the baby (1). Lung development progresses in five distinct stages: embryonic, pseudoglandular, canalicular, saccular, and alveolar (2, 3). Human preterm babies who develop BPD are born in the late canalicular or early saccular stage of lung development. The late canalicular stage is characterized by development of the primitive alveoli and the alveolar capillary barrier, and the differentiation of type I and type II pneumocytes. The early saccular stage is marked by initiation of surfactant production, pulmonary vascularization, and enlargement of terminal airways (2–5). Unique to lung development is the fact that unlike other organs, the lungs complete their development after birth (up to 8 years of age) (6). Alveolar sacs are formed by secondary septation of alveolar ducts. With preterm birth, this programed development is disrupted, and in the setting of inflammation [whether it is due

to infection, mechanical ventilation (MV), or hyperoxia] causes impaired alveolarization leading to BPD. We need to remember that while in sheep, baboons, and humans, the saccular stage occurs *in utero*; in rodent models, it begins at embryonic day 18 and continues through postnatal (PN) day 5 (4, 5).

In spite of many advances in neonatal medicine in the past few decades, like the introduction of better MV strategies and the use of surfactant and antenatal steroids, the incidence of BPD has not declined (7). The incidence of BPD in the United States is about 10,000–15,000 new cases each year out of which the majority of those affected have a birth weight <1250 g (8). Pulmonary and neurodevelopmental sequelae of this devastating disease extend even into adulthood (9).

Genetic (10) and environmental factors (pre- and/or postnatal sepsis, invasive MV, and hyperoxia) (1) act on the preterm human immature lung with inflammation being the common denominator in all these interactions leading to the multifactorial origins of this disease. As shown in **Figure 1**, it is postulated that adverse perinatal exposure/infection with added insults like invasive MV, exposure to hyperoxia, and sepsis causes persistent immune dysregulation. This on top of genetic susceptibility and prematurity leads to persistent inflammation leading to lung remodeling and evolution of BPD.

In this review article, we have attempted to analyze and consolidate current knowledge about the role played by persistent prenatal and postnatal inflammation in the pathogenesis of BPD. We searched PubMed for articles limited to English language with the keywords: "Bronchopulmonary dysplasia or BPD," "inflammation," "chorioamnionitis," "mechanical ventilation," "hyperoxia," "postnatal sepsis," either individually or in combination.

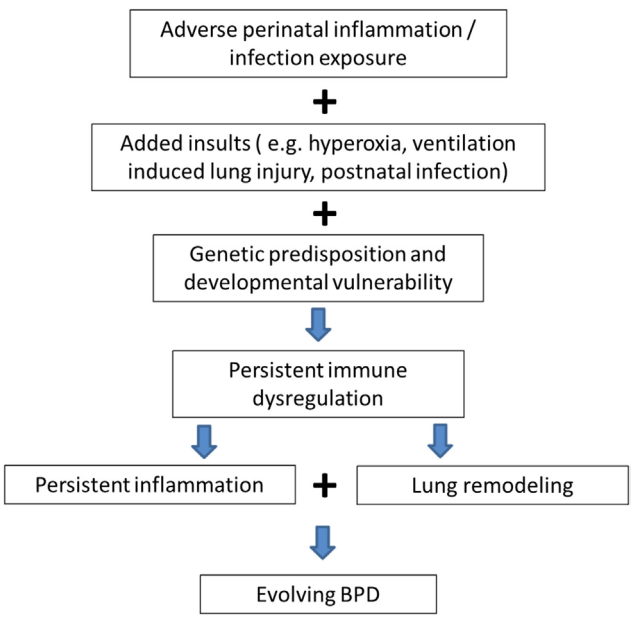

FIGURE 1 | Genetic predisposition and persistent inflammation due to environmental factors (sepsis, invasive mechanical ventilation, and hyperoxia) acting on the foundation of immature lung underlie the pathogenesis of BPD.

We focused on articles published over the last 10 years and used the most relevant ones for this review.

MEDIATORS OF INFLAMMATION IN BPD

Bronchopulmonary dysplasia has been linked to the development of an inflammatory response that can occur in absence of clinical infection. Systemic fetal inflammatory response (11) and neonatal leukemoid reactions (12) have been implicated as a risk factor for BPD. Pulmonary inflammation in BPD is characterized by the presence of inflammatory cells like neutrophils and monocytes, pro-inflammatory cytokines, and other mediators, including soluble adhesion molecules.

The innate immunity and adaptive immunity reinforce each other and act in unison. Cells of the innate immune system secrete cytokines, which can prime lymphocytes thereby modulating adaptive immunity (13). Exposure to a specific antigen causes these primed lymphocytes to have a more rapid and intense immune response (14, 15). Naïve T cells express CD62L (L-selectin) (16). Upon activation, the T cells shed their surface CD62L molecules. In infants with BPD, the expression of the CD62L is decreased on these CD4+ T-cells thereby suggesting T cell activation. CD54 (intercellular adhesion molecule-1 or ICAM-1) is an adhesion molecule that mediates a co-stimulatory signal in T cell activation. CD54 expression is increased upon cell activation (17).

The premature lung is exposed to ongoing oxidative and cellular damage. Damaged lung tissue releases chemotactic factors and inflammatory cytokines, such as interleukin (IL)-1, IL-8 (CXCL-8), and tumor necrosis factor alpha (TNF-α). This leads to an influx of neutrophils and other inflammatory cells with increased release/production of additional pro-inflammatory cytokines (**Table 1**). Multiple studies have shown that IL-1β, IL-6, and IL-8 are elevated very early in the respiratory course of the human preterm population that ultimately develop BPD, and in tracheal aspirates of those with BPD. In contrast, decreased levels of IL-10 in serum and tracheal aspirates have been shown in studies of those infants who developed BPD. In addition to ILs, a large variety of other biomarkers have been detected and associated with the development of BPD in tracheal aspirates, as well as blood and urine samples of premature infants (9, 18). The ones that have been implicated in the animal models include inflammatory cytokines, matrix proteins, growth factors, and vascular factors (9, 18–33). Their role is illustrated in **Table 1**.

We will now describe the major environmental factors that contribute to inflammation, and its persistence, in the pathogenesis of BPD. These include prenatal influences (chorioamnionitis) and postnatal influences, namely early- and late- onset sepsis, invasive MV, and hyperoxia.

PRENATAL FACTORS CAUSING INFLAMMATION – CHORIOAMNIONITIS

As the name suggests, chorioamnionitis is inflammation of the chorion and amnion membranes of the placenta (34).

TABLE 1 | Selected mediators of inflammation, their role, and corresponding expression in BPD.

Mediators of inflammation	Role	Expression in BPD
Inflammatory cytokines		
Interleukins: anti-inflammatory		
IL-10	Suppresses inflammatory response by inhibiting NF-κB	↓/↔
IL-4, IL-13	Suppresses inflammation by inhibiting pro-inflammatory cytokine production	↔
Interleukins: pro-inflammatory		
IL-1, IL-6	Acute phase inflammatory response	↑
IL-8 (CXCL-8)	Main chemoattractant for neutrophils	↑
CC chemokines		
Monocyte chemoattractant protein (MCP)-1, 1α, 1β, 2, 3	Recruit inflammatory cells to area of injury	↑
Macrophage migration inhibitory factor (MIF)	Upstream regulator of innate immune response	↓
Tumor necrosis factor alpha (TNF-α)	Enhances expression of other pro-inflammatory cytokines	↑
Transforming growth factor-beta 1 (TGF-β1)	Pro-inflammatory	↑
Matrix proteins		
Matrix metalloproteinase-8	Disordered pulmonary remodeling after inflammation	↑
Matrix metalloproteinase-9	Pro-inflammatory, interferon-gamma (IFN-γ) signaling	↑
Growth factors		
Endothelin-1	Pro-inflammatory	↑
Vascular endothelial growth factor	Pro-inflammatory	↑/↓
Connective tissue growth factor (CTGF)	Pro-inflammatory	↑
Bombesin-like peptide (BLP)	Increases mast cells in the lung	↑
Breast regression protein-39 (human analog is YKL-40)	Anti-inflammatory	↓
Pulmonary hepatocyte growth factor (HGF)	Alveolar septation, repair	↓
Keratinocyte growth factor (KGF)	Regulates proliferation of alveolar epithelial cells	↓
Miscellaneous		
Interferon-inducible protein 9 (IP-9 – also known as CXCL11)	Pro-inflammatory, IFN-γ signaling	↑
Cyclooxygenase-2 (Cox-2)	Pro-inflammatory, IFN-γ signaling	↑
CCAAT/enhancer-binding protein (C/EBP)	Pro-inflammatory, IFN-γ signaling	↑
Endoglin	Pro-inflammatory	↑
Periostin	Pro-inflammatory	↑
Clara cell secretory protein	Modulates acute pulmonary inflammation	↓
Parathyroid hormone-related protein (PTHrP)	Alveolar growth	↓
Angiopoietin-2	Pro-inflammatory	↑
Lactoferrin	Anti-inflammatory	↓

↑ – *increase;* ↔ – *no change;* ↓ – *decrease.*

Although commonly seen in clinical practice, chorioamnionitis is a complex syndrome associated with pregnancy leading to preterm deliveries (34). Chorioamnionitis has been classified as either histological or clinical. With histological chorioamnionitis, there is infiltration of polymorphonuclear leukocytes and other inflammatory cells like macrophages and T cells as seen microscopically (35–37). Clinical chorioamnionitis is evidenced by fever >37.5°C, uterine tenderness, foul smelling vaginal discharge, abdominal pain, maternal tachycardia with a heart rate >100 bpm, fetal tachycardia HR >160 bpm, and white blood cell (WBC) count >15,000/mm^3 (38, 39).

It has been shown in *in vitro* studies that bacterial products like phospholipase A2, peptidoglycan polysaccharide, proteolytic enzymes, and endotoxins can initiate an inflammatory response. Inoculation of the amniotic cavity with *E. coli* lipopolysaccharide (LPS) or live *Ureaplasma* organisms has been shown to induce structural and functional fetal lung maturation (40–43). Antenatal lung inflammation impacts a variety of signaling pathway regulators like toll-like receptors 2 and 4 (TLR2 and TLR4), growth factors like TGF-β and CTGF, and mesenchymal structural proteins like bone morphogenetic protein-4 leading to vascular remodeling and alveolar simplification, which could be considered akin to a mild BPD phenotype (40–43). However, repetitive LPS exposure and/or chronic chorioamnionitis leads to immune tolerance and a dampened inflammatory response, which in turn allows the lungs to develop close to normal in experimental BPD animals (40–42).

Adverse perinatal outcomes are seen with intra-amniotic inflammation irrespective of the presence of intra-amniotic infection. Colonization *per se*, without inflammation is not associated with adverse outcomes (44). The severity of the adverse outcomes is directly related to the severity of the intra-amniotic inflammation (44). Maternal antibiotic use has been associated with decreased BPD (45).

To summarize, in experimental animals, antenatal inflammation causes lung maturation and some degrees of lung injury, which is modified by the not fully developed innate immune response, exposure to antenatal steroids, and noxious postnatal factors. Not surprisingly, given the variability in definition and impact of various confounding factors, the issue of antenatal inflammation causing BPD in human infants is controversial (42, 46–49). Chorioamnionitis increases the incidence of preterm birth, and if accompanied by lung inflammation could result in surfactant dysfunction allowing for prolonged exposure to supplemental oxygen and invasive MV (11, 48). This "multiple hit" of events could explain the propensity to BPD in such infants (48), though this has not been consistently shown (50). In addition, persistence and non-resolution of lung inflammation lead to BPD by inhibiting secondary septation, alveolarization and normal vascular development, and the compromised ability of the lungs to heal.

POSTNATAL FACTORS CAUSING INFLAMMATION – SEPSIS

Preterm infants are more susceptible to infections since their immune defenses are not fully developed, have vulnerable skin

barrier, and require multiple invasive procedures (51). Postnatal infection/inflammation could either be localized to the lung or could be systemic in origin. Chorioamnionitis increases the risk of early-onset neonatal sepsis, which sets off an inflammatory cascade (48). Also, it has been shown that late-onset sepsis induces a pro-inflammatory and pro-fibrotic response in the preterm lung predisposing it to BPD (51).

Local (intra-tracheal) exposure to LPS (bacterial endotoxin) or dsRNA (a marker of viral replication) in the neonatal rat led to acute cellular and cytokine inflammatory responses, which were associated with histologic features of impaired alveolar development (52, 53).

Neonatal mice injected with intraperitoneal LPS demonstrated reduced lung inflammation and apoptosis after 24 h as compared to adults, and this was associated with activation of the transcription factor, nuclear factor kappa B (NF-κB) (54). Inhibition of NF-κB resulted in increased cell death and alveolar simplification and disruption of angiogenesis via vascular growth factor (VEGF)-R2 (55). It has also been shown that using a targeted deletion of NF-κB signaling (using a lung epithelium-specific deletion of IKKβ – which is a known activating kinase upstream of NF-κB) in a mouse model results in alveolar hypoplasia with decreased VEGF expression (56). In addition, there was increased expression of CXCL-1, as well as its receptor CXCR2. Pretreatment with CXCR2-neutralizing antibody was able to reverse the effects in the developing lung (53). In summary, exposure to either bacterial or viral agents in the rodent model led to features of inflammation, with pulmonary histology suggestive of BPD.

Inflammatory response secondary to viral infections in early post natal stages could be worth considering in the evolution of BPD. Increased neutrophil accumulation, increased expression of CXCL-1 and its receptor CXCR2, and decreased lung alveolarization have been seen with intra-tracheal delivery of viral pro-inflammatory dsRNA in 10-day-old mouse model (53).

POSTNATAL FACTORS CAUSING INFLAMMATION – INVASIVE MECHANICAL VENTILATION

Mechanical ventilation is a risk factor for the development of BPD in premature infants. Lung injury from MV results due to volutrauma, barotrauma, or atelectrauma (57).

When lungs are exposed to high tidal volumes, over distension leads to production of pro-inflammatory cytokines like IL-6, IL-8, and TNFα and reduced expression of anti-inflammatory cytokines like IL-10 (58). Even ventilation at low tidal volumes is deleterious because of the stretch injury it can induce by overdistending partially collapsed lungs. Sustained lung inflation (SLI) has been shown to increase levels of pro-inflammatory cytokines and BPD-like changes in the lungs of preterm lambs (59). There is great need to find non-invasive ventilation strategies for preterm neonates because even "gentle" invasive MV for a shorter duration can induce an inflammatory response (60).

In neonatal rats (7- to 14-day-old – in the alveolar phase of lung development), high tidal volume ventilation increased IL-6 mRNA and upregulated the TGF-β signaling molecule, CTGF mRNA, and protein expression compared to controls (61). In an 8-day-old rat ventilation model, high tidal volumes increased the neutrophilic and inflammatory cytokine mRNA and/or protein expression (IL-1β, IL-6, CXCL-1 and 2) response (62). In a 7-day-old rat model, exposure to MV for 24 h in room air led to cell cycle arrest (63), suggesting a harbinger to alveolar simplification, the pathologic hallmark of BPD.

In an invasive MV model in 2-week-old mice (well into the alveolar phase of lung development) for 1 h, IL-6 lung levels were increased in the high tidal volume ventilation group (64). Studies conducted in 2- to 6-day-old mice (late saccular to early alveolar phase of lung development) ventilated for 8–24 h with room air or 40% O_2 revealed dysregulated elastin (ELN) assembly, a threefold to fivefold increase in cell death, TGF-β activation, and a decrease in VEGF-R2 expression (65, 66). Inhibiting lung elastase activity by using recombinant human elafin or genetically modified mice that expressed elafin in the vascular endothelium was protective of the lung injury (67, 68).

Early studies using a chronically ventilated (3–4 weeks) preterm lamb model of BPD showed evidence of non-uniform inflation patterns and impaired alveolar formation with an abnormal abundance of elastin (69). Inflammation was evident by the presence of inflammatory cells, namely alveolar macrophages, neutrophils, and mononuclear cells and edema (69). In this model, there was also reduced lung expression of growth factors that regulate alveolarization and differential alteration of matrix proteins that regulate ELN assembly (70). A non-invasive (nasal) ventilation approach preserved alveolar architecture (71) and had a positive effect on parathyroid hormone-related protein-peroxisome proliferator-activated receptor-gamma (PTHrP-PPARγ)-driven alveolar homeostatic epithelial–mesenchymal signaling in the preterm lamb model (72).

It has been seen in preterm fetal sheep that there is increased expression of early response gene-1 (Egr-1) as well as pro- and anti-inflammatory cytokines and dynamic changes in heat shock protein 70 (HSP70) (57). This stretch injury also increases expression of granulocyte/macrophage colony-stimulating factor mRNA leading to maturation of lung monocytes to alveolar macrophages (57). Induction of surfactant proteins A, B, and C mRNA is also increased (57). More recently, even short-term stretch injury (15 min) secondary to invasive MV in preterm fetal sheep led to increased levels of pro-inflammatory cytokines, IL-1β, IL-6, monocyte chemoattractant protein (MCP)-1, and MCP-2 mRNA by 1 h (57). This was accompanied by increased presence of inflammatory cells in the bronchoalveolar lavage fluid (BALF) with initial increases in neutrophils and monocytes by 1 h and a transition to macrophages by 24 h (57).

The preterm ventilated baboon model of BPD (delivered at 125 days – at 68% of gestation) showed evidence of alveolar hypoplasia and dysmorphic vasculature, akin to that seen in human BPD (73). Importantly, there were significant elevations of TNF-α, IL-6, IL-8 levels, but not of IL-1β and IL-10, in tracheal aspirate fluids at various times during the period of ventilator support, supporting a role for inflammation (73). In addition, increased matrix metalloproteinase-9 (MMP-9) levels were associated with lung inflammation and edema seen in this invasive ventilation model (74). Alteration of VEGF was also noted in the lungs of

various baboon models (75, 76). Bombesin is a 14-amino acid peptide, initially detected in amphibian skin, but immunoreactive studies have shown the presence of bombesin-like peptide (BLP) in multiple organ systems in mammals (77). In the lung, BLP have been shown to be released by pulmonary neuroendocrine cells (77). BLP blockade improved alveolar septation and angiogenesis in the preterm baboon models (78, 79).

In the 125-day baboon model, treatment with early nasal continuous positive airway pressure (NCPAP) for 28 days led to a pulmonary phenotype similar to 156 days gestational control lungs, suggesting that this non-invasive approach could minimize lung injury (80). In the same model, delayed extubation (till 5 days) versus early extubation to NCPAP at 24 h led to significantly increased BALF IL-6, IL-8, MCP-1, macrophage inflammatory protein-1 alpha (MIP-1α), and growth-regulated oncogene-alpha (GRO-α) in the delayed NCPAP group (81).

Some epidemiological studies showed that replacing invasive MV with NCPAP was associated with BPD reduction (82). No reduction in the incidence of BPD or mortality in the NCPAP group was seen in the COIN study that randomized infants born at 25–28 weeks to receive either NCPAP or intubation with MV in the delivery room (83). The INSURE (IN: intubation, SUR: surfactant, E: extubation) technique has been shown to reduce the need for MV and incidence of BPD (84). Non-invasive ventilation strategies like nasal intermittent-positive pressure ventilation (NIPPV) not only reduce the need for intubation within the first 48–72 h of life, but also have been associated with decreased mortality and/or BPD and hence is a feasible option for the newborn (85–87), though additional studies are required (88). The optimal mode of non-invasive ventilation (for example: type of NCPAP, maximum level of NCPAP, synchronized or non-synchronized method of nasal ventilation), selection of the best nasal interface (short-prongs or mask), and choice of ventilator need to be determined, and this information would be helpful in management of the disease.

To summarize, while the lamb/sheep/baboon ventilation models are in the saccular stage (akin to the human premature babies who are at most risk for BPD at birth), the rat/mouse ventilation models are in the alveolar phase of lung development. However, it is quite obvious that mechanical stretch injury generates an inflammatory response (mostly neutrophils, IL-1β, IL-6, CXCL-1/-2, TGF-β signaling), along with alterations in matrix proteins (ELN, MMP-9) and VEGF. In addition, there is increased cell death and cell cycle arrest. Thus, it appears that an initial inflammatory cascade triggers the signaling of additional molecular mediators that lead to dysregulated vascularization and impaired alveolarization. Interestingly, non-invasive (nasal) ventilation approaches were protective of these responses. Thus, prolonged invasive MV sets off a persistent cascade of inflammatory response that in the setting of hyperoxia takes the "multiple hit" pathway of leading to BPD.

POSTNATAL FACTORS CAUSING INFLAMMATION – HYPEROXIA

Many studies have documented the injurious effects of perinatal supplemental oxygen on lung development. Target levels of O_2 in extremely low birth weight (ELBW) have been studied extensively. The morphologic changes of human BPD resemble hyperoxic lung injury in newborn animals (73). Prolonged exposure to hyperoxia in the neonatal mouse for 14 days or longer results in a phenotype of "old" BPD (89, 90). Exposure to hyperoxia in the critical saccular stage of lung development replicates human BPD, with effects that are dose-dependent on the fraction of inspired oxygen (FiO_2) concentration; the effects last lifelong with increased susceptibility to respiratory tract infections (91–95). Acute lung injury caused by hyperoxia (**Figure 2**) occurs secondary to an inflammatory response, which causes destruction of the alveolar–capillary barrier, vascular leak, influx of inflammatory mediators, pulmonary edema, and ultimately cell death (96). With continued exposure to hyperoxia this inflammatory response and pulmonary edema improve initially but chronic pulmonary inflammation ensues in the following weeks (97). At the cellular level, alveolar or interstitial macrophages express early response cytokines when exposed to hyperoxia, which in turn attract inflammatory cells to the lungs (19).

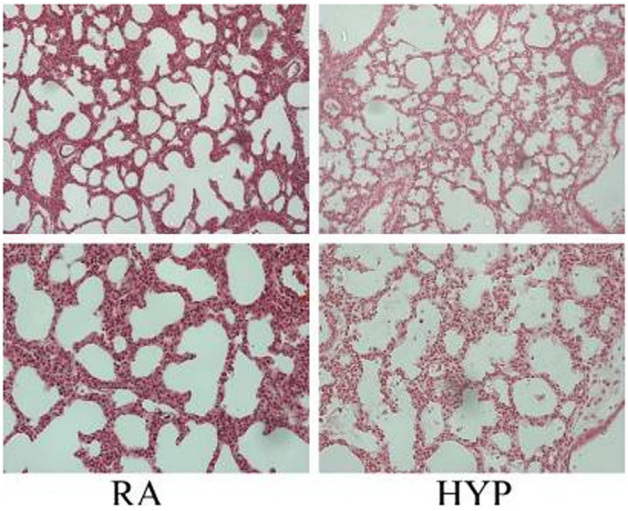

FIGURE 2 | Photomicrographs (×10, upper panel; ×20 lower panel; hematoxylin and eosin stain) of neonatal lung injury noted in newborn mice at postnatal day 2, after 100% O_2 exposure since birth. Note the alveolar exudates and presence of inflammatory cells in the hyperoxia-exposed lungs compared with litter-mate controls in room air. RA, room air; HYP, hyperoxia [with permission from *Semin Fetal Neonatal Med* (2010) 15(4):223–9].

It has been shown that there exists a dose-dependent effect of hyperoxia on severity of BPD in the murine model. Mice exposed to varying concentrations of oxygen ranging from 40 to 100% at PN days 1–4 had more severe disease at higher concentrations of oxygen (92). An oxygen dose-dependent inflammatory response to influenza-A viral infection in adult mice that had been exposed to hyperoxia as neonates has been reported (95). Furthermore, this response was dependent upon the cumulative exposure to oxygen (98).

The specific role of individual inflammatory molecular mediators in the pathogenesis of BPD has been particularly

well illustrated by utilizing lung-targeted overexpressing transgenic models, in room air, resulting in pulmonary phenotypes reminiscent of human BPD. These include IL-1β (99, 100) and IFN-γ (25, 91). In the case of IL-1β transgenic mice, absence of the beta6 integrin subunit was protective of the BPD phenotype (101). Interestingly, inhibition of cyclooxygenase-2 (Cox-2) ameliorated the BPD phenotype in the hyperoxia-induced as well as the IFN-γ lung overexpressing transgenic mouse model in room air. A recent paper has reported that increased Cox-2 activity may contribute to proinflammatory responses in hyperoxia-exposed developing mouse lungs (102).

There is increased expression of IL-1α mRNA in neonatal mice exposed to hyperoxia (89). Lung mRNA for IL-1β also increases in neonatal mice exposed to hyperoxia (103). Transgenic IL-1β overexpression in lung epithelium resulted in BPD phenotype in neonatal mice (100). In hyperoxia-exposed newborn rabbits, the pattern of IL-1β rise and fall matches the rise and fall of histologic inflammation (104). However, in the immature baboon model of BPD, no such pattern between IL-1β levels and inflammation was seen in the tracheal aspirates (73). CINC-1 in premature rat lungs (105) and newborn rabbits (104) exposed to hyperoxia was upregulated. Also, IL-8 levels in tracheal aspirates of the premature baboon model of BPD have been shown to be increased (73).

The lungs of hyperoxia-exposed neonatal mice had no change in IL-10 mRNA expression (103). Also tracheal aspirates of baboon model of BPD show no difference in IL-10 levels (73). IL-1β, IL-6, and IL-8 are pro-inflammatory cytokines and are elevated very early in the course of BPD.

Typically viewed as pro-inflammatory, these cytokines have been shown to be elevated very early in the respiratory course of the human preterm population that ultimately develops BPD (20). Studies have found that serum and tracheal aspirate IL-10 levels were decreased in those infants who developed BPD (20).

A variety of potential therapeutic agents have been used in hyperoxia-exposed mice models that have been shown to decrease inflammation and/or attenuate other parameters of lung injury/BPD phenotype. These include rosiglitazone (106, 107), hepatocyte growth factor (HGF) (108), B-naphthoflavone (109), arginyl-glutamine as well as docosahexaenoic acid (110), and a combination of vitamin A and retinoic acid (111). Treatment with human amnion epithelial cells attenuated some parameters of hyperoxia-induced inflammatory lung injury (mRNA expression of IL-1α, IL-6, TGF-β, platelet-derived growth factor-beta or PDGF-β, mean linear intercept, and septal crest density), but not other aspects, for example, alveolar airspace volume, collagen content, or leukocyte infiltration in neonatal mice (112).

To summarize, while variable initiation and duration of exposure to hyperoxia animal models have been reported as models of human BPD, exposure to hyperoxia for a relatively short (PN1–4) duration in mice, which is at the critical saccular stage of lung development, can result in an inflammatory response sufficient to create the BPD pulmonary phenotype. This can be recapitulated using transgenic mice models of the inflammatory mediators, but kept in room air. Importantly, exposure to 0.4, 0.6, >0.8 FiO_2 can mimic mild, moderate, and severe BPD, respectively. A vast array of therapeutic agents has been reported to be effective in improving alveolar and/or vascular architecture of the hyperoxia-exposed neonatal lung in lambs, rats, and mice.

While hyperoxia exposure is a good starting point for testing the efficacy of potential therapeutic agents, it is important to be able to delineate the responsible molecule/signaling pathway in developmentally appropriate room air models and confirm the results in preventing/ameliorating the BPD phenotype. This would avoid the confounding variable of hyperoxia-induced alterations in multiple other molecular mediators, allowing delineation of targeted molecules in specific signaling pathways for maximal potential therapeutic relevance. Among the inflammatory mediators of hyperoxia-induced lung injury that can mimic the BPD phenotype in room air, the well-defined ones are IL-1β, TGF-β1, CTGF, IFN-γ, and MIF. It would be important to attempt to translate some of the newer targets in specific signaling pathways that have been recently reported, for example, inhibition of Cox-2 (91, 102) as a potential therapeutic option for prevention/amelioration of BPD.

PERSISTENT INFLAMMATION IN BPD

It is important to highlight the fact that for BPD to occur, it requires the known environmental factors to be exposed to the immature lung for a sustained duration, resulting in persistent inflammation. For the chorioamnionitis rodent models, the exposure to LPS is over a few days in the late canalicular/early saccular stage of lung development. For the relative short duration of exposure to invasive MV and hyperoxia in rodent models, 1 postnatal day in the saccular stage of lung development is equivalent to 3–4 weeks in a human preterm infant. Obviously, the larger animal models (sheep/lamb/baboon) also need few days to weeks of injury to develop the pulmonary phenotype of BPD. While some parameters of the early inflammatory response (neutrophils, cytokines such as IL-1, TNFα) may not be detectable after days to weeks of exposure to noxious stimuli, they have already initiated the signaling pathways of the inflammatory process/immune cascade and have affected permanent defects structurally and functionally in the BPD lungs. This is borne out by the facts that the pathologic appearance of large simplified alveoli is permanent following just the first 4 PN days of hyperoxia exposure in mice models (93). Furthermore, these mice have increased mortality when exposed to viral infectious challenge as adults (98, 113). In concordance, preterm neonates with BPD have anatomical and functional pulmonary deficits well into childhood and as adults (114–116). There is some clinical evidence that early interruption of the initial inflammatory response could result in amelioration and potential reversal of these effects (117).

SUMMARY AND CONCLUSION

It is important to remember that while *in vitro* studies are helpful in figuring out the mechanistic significance of a signaling pathway, these are usually conducted with cell lines or freshly isolated single cells of a particular phenotype. Thus, the results of such studies may not accurately reflect the *in vivo* situation of interaction with the multiple cell types found in the lung. In addition, while

the significantly different responses between adult and neonatal lungs to the postnatal factors discussed here – invasive ventilation (118–121), local/systemic sepsis (52, 54, 122–124), and hyperoxia (19, 125–127) – are well established, it is also important to be cognizant of the stages of lung development when comparing animal data for relevance to humans. This is best exemplified by studies that highlight the differential responses in the various stages of lung development (mostly, saccular vs. alveolar) in animal models (99). Furthermore, the degree and duration of exposure to the noxious stimulus (hyperoxia, for example) in the animal models needs to be appropriate in order to attempt to extrapolate the data to humans. For example, a prolonged exposure to hyperoxia from birth to 2 weeks in the mouse, i.e., almost to the end of alveolarization is akin to exposing a preterm neonate to the same to at least up to 2 years of age.

To conclude, it is the preterm lung in the late canalicular/saccular phase of development that is most predisposed to BPD, when exposed to the pre- and postnatal factors. Inflammation and then its persistence in the preterm lung – whether initiated by prenatal factors like chorioamnionitis or whether propagated postnatally with the use of high FiO_2 and invasive MV or sepsis – culminates in BPD. Hence, translational research needs to be aimed at decreasing chorioamnionitis and finding better strategies for early non-invasive MV and optimum use of oxygen for the immature preterm lung for dampening the inflammatory response.

AUTHOR CONTRIBUTIONS

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Commentary: Understanding the Impact of Infection, Inflammation and Their Persistence in the Pathogenesis of Bronchopulmonary Dysplasia

*Jay K. Kolls**

Department of Pediatrics, Richard King Mellon Institute for Pediatric Research, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

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***Correspondence:**
Jay K. Kolls
jay.kolls@chp.edu

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As Drs. Balany and Bhandari (1) point out in their recent review, despite the development of surfactant therapy, and new forms of non-invasive ventilation, bronchopulmonary dysplasia (BPD) remains the most common chronic respiratory disease in newborns/infants. The authors point out the key drivers of disease including infection, barotrauma, hyperoxia, and inflammation and highlight the potential role that inflammation plays in driving the disease. Lung inflammation can be triggered by activation of the innate immune system and this can be triggered by cell surface or cytoplasmic receptors that fall into the three key categories—toll-like receptors (TLRs), Nod-like receptors (NLRs), and c-type lectin receptors (CLRs). TLRs 1, 2, 4, and 6 are surface receptors that recognize both exogenous stimuli such as lipopeptides or endotoxin (2) and endogenous ligands such as Hsp70 or oxidized phospholipids (3). Thus, inflammation in BPD could be driven by ligands from invading pathogens in an intubated patient or endogenous ligands that could be released during hyperoxia. One could envision that the therapeutic approach may be different given the proximal driver of inflammation. Thus, it is important to not only to characterize the type of inflammation but we need a keen understanding of what initiated the response. NLRs are cytoplasmic receptors and have several ligands including muramyl dipeptide (2). These receptors play a key role in activation of the inflammasome which is an intracellular protein structure that results in caspase-1 mediated cleavage of proIL-1β or pro-IL-18 into mature secreted proteins. By contrast, other IL-1 family members such as IL-1α or IL-33 can be released and be biologically active independent of caspase-1-mediated cleavage. This pathway is an active area of drug development as there is strong genetic evidence of IL-33 in asthma (4, 5). For ligands that bind IL-1R1, IL-1β, and IL-1α, Anakinra has been FDA approved for the treatment of rheumatoid arthritis and for the treatment of neonatal-onset multisystem inflammatory disease (5). Given that overexpression of IL-1β results in decreased alveolarization (6), this may be a viable target. However, this pathway is also critical for host immunity to several pathogens including influenza (7) and *Staphylococcus aureus* infection (8), so one would need to proceed with caution and likely exclude patients with active infection. CLRs recognize carbohydrate ligands such as mannans and glucans and drive inflammation but also can be involved in resolution of inflammation as well (9).

As Drs. Balany and Bhandari state in their review (1), cytokines have been extensively evaluated in BPD including IL-1, IL-6, TNF, and IL-10. Moreover the CXCR2 ligands, CXCL1, and CXCL8 have also been found to be elevated in BPD. In preclinical models, CXCR2 antagonism appears to improve lung histology (10). CXCR2 antagonists have been studied in human clinical trials in cystic fibrosis and COPD. A recent phase 2 study showed an increased in FEV1 in COPD (11), and another trial showed reduced inflammation in sputum biomarkers in CF (12). Thus, this may be an approach in BPD. In the adult lung, CXCL8 can be made by both alveolar and tissue macrophages as well as the lung epithelium. It has been recently show in neonates that γδ T-cells are a major source of CXCL8 (13) and thus as the sources of CXCL8 may be unique in the neonatal lung—this will need to be taken into consideration for rational drug approaches.

Finally is the issue of resolution of inflammation. Namely, is BPD due to a failure to resolve inflammation? To this end, a critical area of needed research in this regard is the role of lipid mediators such as resolvins and lipoxins in BPD (14). Lipoxin A4 has been shown to be reduced in chronic neutrophilic lung inflammation in CF (15). Thus, in addition to cytokine measurement, there needs to be more research in assaying both pro-inflammatory and pro-resolving lipid mediators in the lungs of BPD subjects to determine if this may also be a target for therapeutic intervention. Moreover, we need more basic understanding of how gestational age and lung maturity affect the inflammatory response in the lung—both in terms of intitiating a response and in it's resolution.

In conclusion, BPD is clearly a disease associated with airway inflammation with likely a complex series of initiators. However, defining inflammation—using unbiased omic approaches (proteomics and lipidomics) as well as replication studies in well-characterized patient cohorts may improve our understanding of the potential role of inflammation in disease pathogenesis and open up new avenues of therapeutic intervention.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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The Extracellular Matrix in Bronchopulmonary Dysplasia: Target and Source

*Ivana Mižíková[1,2] and Rory E. Morty[1,2]**

[1] Department of Lung Development and Remodelling, Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany, [2] Pulmonology, Department of Internal Medicine, University of Giessen and Marburg Lung Center, Giessen, Germany

Bronchopulmonary dysplasia (BPD) is a common complication of preterm birth that contributes significantly to morbidity and mortality in neonatal intensive care units. BPD results from life-saving interventions, such as mechanical ventilation and oxygen supplementation used to manage preterm infants with acute respiratory failure, which may be complicated by pulmonary infection. The pathogenic pathways driving BPD are not well-delineated but include disturbances to the coordinated action of gene expression, cell–cell communication, physical forces, and cell interactions with the extracellular matrix (ECM), which together guide normal lung development. Efforts to further delineate these pathways have been assisted by the use of animal models of BPD, which rely on infection, injurious mechanical ventilation, or oxygen supplementation, where histopathological features of BPD can be mimicked. Notable among these are perturbations to ECM structures, namely, the organization of the elastin and collagen networks in the developing lung. Dysregulated collagen deposition and disturbed elastin fiber organization are pathological hallmarks of clinical and experimental BPD. Strides have been made in understanding the disturbances to ECM production in the developing lung, but much still remains to be discovered about how ECM maturation and turnover are dysregulated in aberrantly developing lungs. This review aims to inform the reader about the state-of-the-art concerning the ECM in BPD, to highlight the gaps in our knowledge and current controversies, and to suggest directions for future work in this exciting and complex area of lung development (patho)biology.

Keywords: bronchopulmonary dysplasia, extracellular matrix, hyperoxia, mechanical ventilation, collagen, elastin, lung development

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****Correspondence:***
Rory E. Morty
rory.morty@mpi-bn.mpg.de

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BRONCHOPULMONARY DYSPLASIA IN CONTEXT

The lung is the key organ of gas exchange in air-breathing mammals. This gas exchange structure is derived from the primitive foregut and proceeds through a phase of early (embryonic) development (1–3), when the conducting airways and conducting vessels are generated and organized (4). Early lung development initiates with the embryonic stage that occurs 4–7 weeks post-conception in humans [embryonic day (E)9–E12 in the mouse]. The embryonic stage is followed by the pseudoglandular stage, which occurs at 5–17 weeks post-conception in humans (E12–E17 in mice). The final stage of *early lung development* is the canalicular stage, occurring at 16–26 weeks post-conception

in humans (E17–E18 in mice), at which point, the process of alveolarization begins, which is characterized by the thinning of the interstitial tissue (**Figure 1**). This marks the beginning of *late lung development*, where the distal airways then form saccular units in the saccular stage, which is evident in humans at 24–38 weeks post-conception [E18-post-natal day (P)4 in mice], and these saccular units are divided by secondary septa (the process of "secondary septation") during the alveolar stage, which is evident at 36 weeks post-conception to 36 months post-natal (and beyond) in humans (P4–P28 in mice). The objective of late lung development is the production of a large number of small alveoli, the principal gas exchange units of the lung. This process, which is poorly understood, creates a large surface area over which gas exchange takes place. Current knowledge on late lung development implicates transcription factors and epigenetic effects, which together regulate genetic programs driving lung development. These programs work in concert with contact- and growth factor-mediated cell–cell communication (5–7) to drive lung development. The development of the lung is also driven in part by physical forces from breathing motions and the production and remodeling of the extracellular matrix (ECM) scaffold.

Multiple diseases are complicated by disturbances to lung development. Notable among these is bronchopulmonary dysplasia (BPD), which affects prematurely born infants with acute respiratory failure that receive oxygen therapy, first described by William (Bill) Northway and colleagues in 1967 (10, 11). While oxygen supplementation is a life-saving intervention, the associated oxygen toxicity stunts the post-natal development of the lung. This damage to the developing lung is exacerbated by barotrauma and volutrauma caused by positive-pressure mechanical ventilation, and also by inflammation. Affected infants exhibit blunted lung maturation, and BPD represents a significant cause of morbidity and mortality in a neonatal intensive care setting (11–14). Longitudinal studies suggest that disease sequelae persist into adult life (15–17). Examinations of autopsy material from patients that have died with BPD have formed the basis of hypotheses about pathogenic processes at play that limit alveolarization. These observations include (i) severe disturbances to the development of the pulmonary vasculature (18), (ii) changes in the cellular structure and composition of the developing alveolar units, (iii) increased proteolysis in the alveolar compartments, (iv) increased inflammatory cell infiltration, (v) deregulated growth factor signaling, and (vi) perturbations to the ECM architecture of the developing lung: most notably, the abundance and organization of collagen and elastin fibers (19–22). These disturbances have also been noted in animal models of BPD (23, 24).

It is the objective of this review to highlight key observations made regarding changes to the ECM architecture of the lung – both in clinical BPD and in experimental animal models of BPD (referred to herein as "experimental BPD") – and to integrate these observations into a pathogenic pathway. Furthermore, attention will be paid to current controversies in the field, and also, to the key gaps in our knowledge, where urgent additional work is still to be undertaken.

EARLY STUDIES: THE ECM IN LUNG DEVELOPMENT AND BPD

The ECM represents a very complex network of structurally, mechanically, and biochemically heterogeneous components (25). The components include the classic "players": collagen and elastin, which constitute 50% (26) and 18% (27), respectively, of the lung ECM. This list continues to grow, with fibrillin (28) and fibulin (29) glycoproteins, and integrin receptors of ECM components (30) being more recent additions. The ECM serves as a scaffold that directs lung development, and the ECM structure itself is continuously remodeled as lung development proceeds (31, 32). As such, the production of ECM components, as well as the systems that regulate the deposition and stability of the ECM, must be considered. These systems include chaperones and enzymes that catalyze the post-translational processing of ECM components, as well as systems that destabilize and degrade the

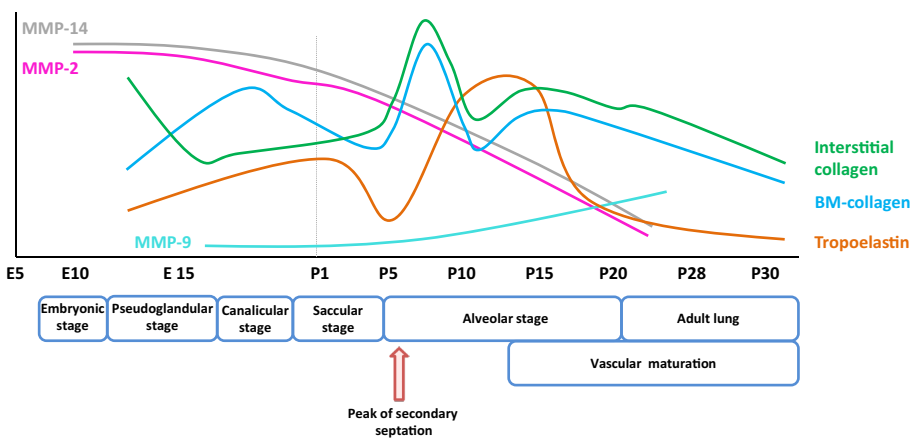

FIGURE 1 | Expression pattern of selected extracellular matrix components and remodeling enzymes over the course of early and late lung development in mice. The trends illustrated represent a synthesis of the data presented in several publications (8, 9), and span the embryonic and post-natal lung maturation period. Abbreviations: BM, basement membrane; E, embryonic day; MMP, matrix metalloproteinase; P, post-natal day.

ECM to facilitate ECM renewal. The remodeling of collagen during lung development has been recognized since the early 1970s (33). Pioneering work by Ron Crystal's group identified the heterogeneity of fibrillar collagens in the lung during lung development (34–36). Observations on the dynamic remodeling of other collagen types, including basement membrane-type IV collagen then followed, in the context of early lung development (37, 38). Similarly, pioneering work by Janet Powell and Philip Whitney in the early 1980s described changes in lung elastin levels as post-natal lung development proceeded (39). Subsequent work by Ron Crystal (40) and Robert Rucker's (41) teams highlighted the dynamic expression of tropoelastin during the course of post-natal lung development. Building on these studies, early work demonstrating that lathyrogens could disturb normal lung development (42) highlighted the role of collagen and elastin in the post-natal maturation of the lung. Soon to follow these reports were key observations of perturbed ECM structures in disorders of lung development. Leading work by Donald Thibeault and William Truog, in particular, identified secondary collagen fibers in the developing parenchyma of neonates with BPD that were "disorganized, tortuous, and thickened" (20, 21). Similarly, elastin fibers exhibited an abnormal structure in infants with BPD, both in the parenchyma (43–45) and in the vasculature (46). These early studies firmly established a role for proper lung ECM homeostasis in normal lung development, and described severe structural perturbations to the lung ECM that accompanied aberrant lung development. Clearly, it is important to note that it is sometimes difficult to establish whether the perturbations to ECM structure noted in clinical subjects with BPD are a cause of aberrant alveolarization, or a consequence of blunted lung development. This applies equally to pathological material from animals in which BPD has been modeled. Since these initial studies reported above, many strides have been made in our understanding of the production of the structural components of the ECM during post-natal lung development, which will be considered in detail below.

KEY STRUCTURAL COMPONENTS OF THE ECM: COLLAGEN AND ELASTIN

Collagen

Collagen is the most abundant protein within the interstitial ECM. In the lung, collagen fibers [represented predominantly by the fibrillar collagens, collagen type I and III produced by fibroblasts (**Table 1**)] are found in the bronchi, blood vessels, and the alveolar septa (35, 47, 48). The abundance of lung parenchymal collagen increases over the course of lung development. In mice,

TABLE 1 | Cellular localization and origin of individual components of extracellular matrix and extracellular matrix remodeling enzymes.

Extracellular matrix component	Origin/source				
	Epithelial cells	Fibroblasts	Endothelial cells	Smooth muscle cells	Inflammatory cells
Collagen		(51–53)			
EC-SOD	(54, 55)				
Elastin		(51, 53, 56)		(51, 57)	
Fibrillin-1		(58, 59)			
Fibronectin		(58, 60–62)	(60)	(60)	(60, 61)
Fibulin-5/DANCE		(63)		(64)	
Heparan sulfate	(65)	(66)			
Integrins	(67–71)	(69)	(70)	(70)	
LOX		(72, 73)		(72, 73)	
LOXL1		(72, 73)		(72, 73)	
LOXL2		(72, 73)		(72, 73)	
LTBP2		(58)			
MMP-1	(74)	(74)			
MMP-14/MT1-MMP	(74–76)	(75)	(76)		
MMP-2	(74, 75, 77, 78)	(75)	(74)		(77)
MMP-9	(74, 77–79)	(61)			(77, 79)
PLOD1	(80)	(80)	(80)	(80)	
PLOD2	(80)	(80)	(80)	(80)	
PLOD3	(80)	(80)	(80)	(80)	
Tenascin C	(81, 82)	(81–84)	(83, 84)	(83)	(81)
TGF-β	(78)	(61)			
TGM2/tTG	(85–87)	(52, 85, 86)	(85)	(85)	
TIMP-1	(75)	(75)			
TIMP-2	(74, 75)	(75)	(74)		

Numbers in parentheses indicate the citations reporting the identification of the extracellular matrix components or remodeling enzymes in the indicated lung cell types. The citations are not all inclusive, and represent only a selection of reports.

EC-SOD, extracellular superoxide dismutase 3; LOX, lysyl oxidase; LoxL1, lysyl oxidase-like 1; LoxL2, lysyl oxidase-like 2; LTBP2, latent TGF-β-binding protein 2; MMP, matrix metalloproteinase; MT1, membrane-type 1; PLOD, procollagen-lysine, 2-oxoglutarate 5-dioxygenase; TGF-β, transforming growth factor-β; TGM2, transglutaminase 2; TIMP, tissue inhibitor of metalloproteinase; tTG, tissue transglutaminase.

gene expression of fibrillar collagens *Col1a1* and *Col3a1*, as well as basement membrane collagens *Col4a1* and *Col4a2*, is reported to have peaked at P7 (**Figure 1**). By this time, the collagen had formed a delicate interstitial network of fibers that could aid the process of alveolar development (8). While *Col1a1*$^{-/-}$ mice, which lack collagen Iα_1, died *in utero* due to the rupture of major blood vessels, no abnormalities were noted in lung branching morphogenesis in these mice (49, 50). However, elevated levels of other fibrillar collagens, including collagen III and V levels, were noted in *Col1a1*$^{-/-}$ mouse embryos, suggesting a possible compensation for the loss of functional collagen I (50).

Alterations to the structure and integrity of collagen networks have been reported in several animal models of BPD and emphysema, which are diseases of the lung parenchyma that represent a failure of alveolar formation and the destruction of existing alveoli, respectively (21, 72, 88). Studies in various BPD animal models have revealed increased collagen production (**Table 2**), thickened collagen fibers, and increased rigidity of the lung (72, 88, 89) to be associated with experimental BPD. This is consistent with observations made in clinical subjects, where an increased number of collagen-positive cells, elevated levels of the fibrillar collagens, collagen I and collagen III were observed; and BPD patients revealed a specific increase in the collagen I/collagen III ratio (48, 90). Furthermore, elevated levels of collagen IV (91) have been noted in bronchoalveolar lavage (BAL) fluids from patients with BPD. These observations are supported by microscopic studies on patient tissues. Thibeault and colleagues (21) observed thickened and disorganized collagen fibers, and a generally damaged collagen network in the lungs of infants diagnosed with BPD after positive-pressure ventilation. It was proposed by those investigators that enlargement of alveoli due to ventilation leads to compression of surrounding ECM structures and damage to the collagen and elastin niche, disturbing the normal septation process. However, both adult rats (92) and newborn mice (93) exposed to sub-lethal normobaric hyperoxia up-regulated collagen I production, assessed by northern blot and immunoblot, respectively. In the case of newborn mice, the increased collagen I production was attributed to activation of the pro-fibrotic growth factor, transforming growth factor (TGF)-β, which stimulated collagen production and secretion by fibroblasts. Increased collagen deposition in the lung parenchyma of newborn mice has been confirmed in the hyperoxia-based mouse BPD model by picrosirius red staining (72, 94). Additionally, total lung collagen protein levels were increased by 63% after exposure of developing mouse pups to hyperoxia (89). Taken together, these reports make a strong case for dysregulated collagen expression in aberrant lung development associated with clinical and experimental BPD.

Collagen production under physiological and pathophysiological conditions is regulated by inter alia growth factors, such as TGF-β, where *in vitro* stimulation of primary lung fibroblasts drives *Col1a1* production (95, 116). This is significant, because elevated TGF-β levels were associated with BPD in preterm infants (115). TGF-β has also been causally implicated in the blunted alveolar development associated with hyperoxia exposure in the mouse hyperoxia model of BPD (117). The connection between TGF-β and collagen deposition in the developing lung is noteworthy. Over-expression of TGF-β driven by the *Scgb1a1* (encoding surfactant-associated protein C, pro-SPC) promoter in a doxycycline-inducible system is sometimes used as an animal model of BPD. Over-expression of TGF-β in this model not only resulted in blunted alveolarization but also increased deposition of collagen in the developing septa (118). Furthermore, over-expression of TGF-β in the developing lung *in utero* caused pulmonary hypoplasia that was accompanied by thickening of the collagen fibers and excessive collagen deposition in the septa (119). Exactly how the blunted alveolarization connects with perturbed ECM generation, both of which are guided by TGF-β, remains to be clarified.

Failed alveolar septation in both clinical and experimental BPD is clearly accompanied by changes to collagen production and deposition in the lungs. Studies, to date, have addressed primarily the fibrillar collagens collagen I and collagen III, however, the remaining 26 other collagens have received little or no attention. It remains of interest to explore whether perturbations to the expression of those collagens might be associated with arrested alveolar development. Similarly, no studies, to date, have examined the regulation or activity of the procollagen processing proteases, bone morphogenetic protein 1 (BMP-1) and ADAM metallopeptidase with thrombospondin type 1 motif, 2 (ADAMTS2). Both enzymes are required for procollagen processing and assembly into fibrils, during lung development.

Elastin

Elastic fibers consist of extensively cross-linked elastin and fibrillin (28) microfibrils. These structures are associated with accessory molecules, including latent TGF-β-binding protein (LTBP), microfibril-associated proteins, fibulin, emilin, and microfibril-associated glycoprotein (MAGP) family members. Elastin fibers are located throughout the developing lung, in the developing conducting airways and alveolar ducts, the conducting vessels, and the developing septa. As illustrated in **Figure 1**, the expression of elastin in mice is dynamically regulated over the alveolarization period. Elastin expression dramatically increases at a time-point coincident with the "burst" of secondary septation that drives the formation of the alveoli. Elastin expression remains high throughout the secondary septation period [for example, in mice, over (P5–P15)] and rapidly decreases once alveolarization has been completed (8, 120). However, reactivation of elastin expression occurs in adult lungs under pathological conditions, such as emphysema and pulmonary fibrosis, where disorganized elastic fibers have been described (22, 120). The first hints that elastin plays a role in lung development included the observations that lung elastin levels were modulated as post-natal lung development proceeded (39). Additionally, the expression of tropoelastin, the "elastin monomer," was dynamically regulated over the course of post-natal lung development in rodents (40, 41). During lung alveolarization, elastin is specifically deposited in "foci" at the tips of developing septa, suggesting a role in the process of secondary septation, which generates the alveoli. The spatially regulated deposition of elastin that coincides with secondary septation has led to the idea that elastin is a driver of lung development (121–123).

Further support for a role for elastin in lung development has been obtained using elastin-deficient mice. Elastin deficiency

TABLE 2 | Dysregulation of the expression of extracellular matrix components and remodeling enzymes in clinical bronchopulmonary dysplasia and experimental animal models.

ECM component	Expression in the disease/experimental condition		
	Bronchopulmonary dysplasia	Hyperoxia	Mechanical ventilation
Collagen	↑ (48, 91)	↓ (Fibroblasts, *in vitro*) (95) ↑ (Mouse) (72, 89, 93) ↑ (Rat) (92)	
EC-SOD		↓ (Mouse) (55)	
Elastin		↓ (Fibroblasts, *in vitro*) (95) ↓ (Mouse) (89, 96) ↑ (Mouse) (51, 72, 93, 97) ↑ (Rat) (98)	↑ (Mouse) (23, 99–101) ↑ (Lamb) (24, 102) ↑ (Rat) (103)
Fibrillin-1		↑ (Mouse) (51)	↑ (Mouse) (99) ↑ (Lamb) (24)
EMILIN-1		↑ (Mouse) (23, 72)	↓ (Mouse) (23)
Fibrillin-2		↑ (Mouse) (51)	↓ (Mouse) (23, 99)
Fibronectin	↑ (60, 62, 104, 105)	↑ (Mouse) (105) ↑ (Rabbit) (106)	
Fibulin-5/DANCE		↑ (Mouse) (51, 72)	↓ (Mouse) (23) ↑ (Rat) (103) ↑ (Lamb) (24)
Integrins		↑ (Mouse) (51)	
Lox	↑ (72)	↑ (Mouse) (51, 72, 89)	↑ (Mouse) (23) ↑ (Lamb) (24)
LoxI1	↑ (72)	↑ (Mouse) (72, 89)	↓ (Mouse) (23) ↑ (Lamb) (24) ↑ (Rat) (103)
LoxI2		↑ (Mouse) (72, 89)	
MMP-1		↑ (Rat) (92)	↓ (Baboon) (107)
MMP-16		↓ (Rat) (108)	
MMP-2	↓ (109)	↓ (Rat) (110) ↕ (Rat) (78) ↑ (Rat) (77) ↑ (Mice) (93)	
MMP-8	↑ (111, 112)		↓ (Baboon) (107)
MMP-9		↓ (Rat) (110) ↕ (Rat) (78) ↑ (Rat) (77) ↑ (Mice) (93)	↑ (Rat) (103) ↑ (Mouse) (100, 101) ↑ (Baboon) (107)
MMP-9:TIMP-1	↑ (113, 114)		↑ (Baboon) (107)
MT1-MMP		↑ (Rat) (78)	
PLOD1		↑ (Mouse) (80)	
PLOD2	↑ (80)	↑ (Mouse) (80)	
PLOD3		↑ (Mouse) (80)	
Tenascin C	↑ (83)	↓ (Fibroblasts, *in vitro*) (95)	↑ (Rat) (103)
TGF-β	↑ (115)	↑ (Mouse) (51) ↑ (Rat) (78, 92)	↑ (Lamb) (24)
TIMP-1	↓ (113)	↑ (Fibroblasts, *in vitro*) (95) ↑ (Rat) (78, 110)	
tTG	↑ (85)	↑ (Mouse) (85)	

Arrows indicate the direction of dysregulated expression: ↓, down-regulation; ↑, up-regulation; ↕, temporal regulation in either direction over time.
ECM, extracellular matrix; EC-SOD, extracellular superoxide dismutase 3; EMILIN-1, elastin microfibril interfacer 1; LOX, lysyl oxidase; LoxL1, lysyl oxidase-like 1; LoxL2, lysyl oxidase-like 2; LTBP2, latent TGF-β-binding protein 2; MMP, matrix metalloproteinase; MT1, membrane-type 1; PLOD, procollagen-lysine, 2-oxoglutarate 5-dioxygenase; TGF-β, transforming growth factor-β; TGM2, transglutaminase 2; TIMP, tissue inhibitor of metalloproteinase; tTG, tissue transglutaminase.

is accompanied by perinatal lethality, and *Eln*$^{-/-}$ mice exhibit arrested perinatal development of the terminal airway branches, and enlarged terminal air sacs (124). Elastin haploinsufficient (*Eln*$^{+/-}$) mice, which express 50% of the elastin seen in wild-type mice (125), exhibited normal lung development and normal alveolar structures, although there is some evidence that the elastin deposition in *Eln*$^{-/-}$ mice was abnormal (99). Modulating the dose of elastin to <50%, by expressing the human elastin gene in a transgenic homozygous-null *Eln*$^{-/-}$ mouse strain reduced elastin levels to 37% of wild-type mouse levels. While transgenic expression of human elastin rescued the perinatal lethality observed in *Eln*$^{-/-}$ mice, a pronounced blunting of alveolar development was noted (125). These data indicate that a baseline threshold of elastin abundance is required for normal lung development to proceed. All of these observations underscore important roles for the correct spatio-temporal production of elastin structures in the developing lung.

In the context of lung disease, abnormal elastin fiber structures have been observed in the parenchyma of aberrantly developing lungs from prematurely born ventilated neonates (126). Parallel trends have been observed in animal models of BPD, where in response to mechanical ventilation or perinatal exposure to hyperoxia, the normally organized deposition of elastin fibers into foci at the tips of developing septa is lost. Rather, elastin fibers are noted in the walls (not the tips) of the thickened developing septa and have been described to be "brush-like," "thickened," and "loose" (32, 102, 127–129).

The pathological mechanisms behind the disturbed production and deposition of elastin in aberrantly developing lungs remains to be clarified, however, much work in this area has been already done, and remains ongoing. There is a body of evidence that suggests that expression of the *Eln* gene is up-regulated by hyperoxia in animal models of BPD, as revealed by real-time reverse transcription (RT)-polymerase chain reaction (PCR) analysis of mRNA pools from lung homogenates (51, 72, 97). The cell types reported to produce elastin in the lung are listed in **Table 1**, which include fibroblasts and smooth muscle cells. How hyperoxia modulates *Eln* gene expression might be attributed to growth factor stimulation or inhibition of elastin synthesis. Both TGF-β (130, 131) and insulin-like growth factor (IGF) (132) stimulated *Eln* gene expression, whereas some forms of platelet-derived growth factor (PDGF) suppressed *Eln* gene expression (133). Furthermore, the stability of *Eln* mRNA was increased by TGF-β, without impacting mRNA synthesis by lung fibroblasts (134). This is important, since increased TGF-β signaling and levels of TGF-β ligands were associated with experimental (117) and clinical BPD (115). Apart from TGF-β, increased IGF levels were also associated with experimental (135) and clinical (136) BPD, whereas decreased levels of some forms of PDGF were associated with clinical BPD (137). Taken together, these data would suggest that the pro-elastogenic effects of TGF-β and IGF were promoted, while the anti-elastogenic activity of PDGF was blocked during arrested alveolarization associated with BPD. These effects may also explain the increased abundance of *Eln* mRNA in the lung in hyperoxia-based experimental animal models of BPD.

It might be argued that given the extraordinarily long half-life of elastin fibers in the lung [estimated to be several years in the mouse (138)], studies on gene expression are less meaningful than studies on elastin protein production and organization into elastic fibers. Experimental studies on alveolarization tend to examine elastin distribution by light microscopy [for example, with Hart's stain (72, 100, 101) or immunohistochemistry (51)], and infer elastin abundance from those studies. However, some studies have directly addressed insoluble elastin fiber abundance biochemically, where, in contrast to elevated mRNA levels, there appeared to be a paucity of insoluble elastin in affected lungs, assessed by lung desmosine or isodesmosine amounts (89, 96). The paucity of elastin was generally accompanied by the clearly disorganized structure and distribution of elastin fibers evident in the developing septa. This discord between elastin gene expression (which was increased) and the abundance of insoluble elastin (which was decreased) in injured developing lungs (together with perturbed elastin fiber structure and distribution) has several possible explanations, none of which have yet been experimentally tested. (i) The post-transcriptional regulation of *Eln* gene expression may be affected. For example, translation of mature *Eln* mRNA may be blocked by microRNA species generated in response to hyperoxia. Among the microRNA species that have been identified the target elastin are miR-29a/b/c (139) and miR-184, miR-194, miR-299, and miR-376b (http://www.mirbase.org). The possibility of microRNA regulation of elastin expression in the lung has not yet been addressed. Alternatively, the paucity of insoluble elastin in the background of increased *Eln* mRNA abundance might be attributed to (ii) defective post-translational maturation of elastin during fiber formation, or (iii) increased proteolytic degradation of elastin. Concerning post-translational maturation of elastin fibers, many accessory proteins have been identified that can associate with elastin fibers. These include the glycoproteins emilin (140), fibulin (29), LTBP (141), and MAGP family members (142). Discordant expression of these elastin fiber-associated proteins may result in unstable or malformed fiber structures. Indeed, Richard Bland has proposed that the uncoupling of elastin synthesis and assembly is a pathogenic contributor to disordered elastin fiber generation in BPD (23). Elastin fibers with abnormal physical properties may also result from the aberrant activity of the elastin maturation machinery, including the hydroxylation and cross-linking activities of lysyl hydroxylases and lysyl oxidases, respectively. These possibilities are discussed below. Alternatively, changes in the proteolytic capacity of injured, developing lungs may impact elastin fiber production or turnover, either directly (by proteolysis) or indirectly (by regulating the activity of mediators of elastin production). It is these lines of enquiry that are likely to further our understanding of *why* elastin organization is disturbed, and *what* impact this has on alveolarization in the developing lungs.

Some reports addressing the role of serine proteinases in the regulation of elastin production have already yielded exciting data. The group of Richard Bland has examined the utility of blocking serine peptidase activity in the context of BPD. Serine peptidase activity, such as that of neutrophil elastase, was elevated in the lung in clinical and experimental BPD. Mechanical ventilation of mouse pups with 40% O_2 increased elastin degradation and disturbed septal elastin fiber deposition in the mouse lung, which was prevented by intratracheal administration of the

neutrophil elastase inhibitor elafin (100). Thus, inhibition of neutrophil elastase activity [and probably matrix metalloproteinase (MMP)-9 activity as well, since MMP-9 can also be inhibited by elafin] partially restored proper elastin structures and improved lung alveolarization in this model. Furthermore, inhibition of neutrophil elastase activity blunted inflammation and inhibited the generation of active TGF-β that was proposed to be released from the ECM by proteolysis. In support of this idea, transgenic over-expression of elafin in the vascular endothelium similarly protected mice against the aberrant alveolarization and perturbed elastin assembly caused by mechanical ventilation (101). Subsequent exciting work by Keith Tanswell's group has similarly reported that neutrophil elastase inhibition with sivelestat also improved lung structure and elastin deposition in the hyperoxia-based BPD animal model in mice (98). In this study, it is also noted that administration of anti-elastin antibodies in the mouse hyperoxia model of BPD prevented inflammatory infiltration into the lungs. Thus, these investigators raised the exciting possibility that neutrophil elastase-generated elastin fragments acted as pro-inflammatory matrikines (143), suggesting a mechanism by which hyperoxia exposure provoked lung inflammation. These data also raise further questions, for example, while neutrophil elastase inhibition clearly improved alveolarization in two different animal models of BPD, the underlying mechanisms remain unclear. The organization of elastin fibers was improved in both models, and inflammation and TGF-β activation was blunted. However, it remains unclear whether the improved alveolarization was a direct or indirect consequence of elastase inhibition (144). For example, was the generation of elastin fragments sufficient to provoke lung inflammation, or did the elastase-mediated activation of TGF-β play a role in this process as well? Elastase inhibition in the background of TGF-β neutralization would go some distance to resolving these open questions.

One vexing controversy in the lung alveolarization field is: are elastin protein levels elevated or reduced in the aberrantly developing lungs in the hyperoxia-based animal models of BPD? In mechanically ventilated lambs and mice, multiple reports document increased *Eln* mRNA levels, which were consistent with increased elastin protein levels in the lung (23, 102). However, this was not the case with normobaric hyperoxia-based models in mice, where many reports also confirm that Eln mRNA levels were up-regulated by hyperoxia exposure, but there appeared to be a paucity of lung insoluble elastin, when (iso)desmosine was used as a surrogate for mature, insoluble elastin fibers (89, 96). However, these observations are complicated by other reports of *increased* elastin protein in the hyperoxia models, employing either slot–blots (98) or immunoblots (51, 93). This controversy must still be resolved. These discordant data might be attributable to the methodology employed, where protein extraction by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) for the blot-based protocols may have a different capacity for the extraction of insoluble elastin compared with the whole-lung hydrolyzates used in the (iso)desmosine approaches. Irrespectively, neither approach address the quantification of elastin specifically in the developing septa, which represents a major limitation of all of the approaches currently employed.

The current state of the field seems to suggest less lung elastin and more lung collagen, at least in the hyperoxia models of BPD. Given that, collagen imparts rigidity and elastin imparts elasticity to the lung, a shift in the collagen:elastin ratio may impact alveologenesis. This shift in collagen:elastin ratio may be as much as threefold increased by hyperoxia exposure (89). This is likely to dramatically impact lung compliance, and given the importance of the physical forces generated by breathing motions in "pulling the alveoli into shape," a shift in the lung collagen:elastin ratio cannot be discounted as a possible contributing factor to lung alveolar development.

ADDITIONAL STRUCTURAL COMPONENTS OF THE ECM

Fibrillins

Fibrillins are elastin-binding glycoproteins (**Figure 2**) that make up the bulk of the microfibril component of elastic fibers, and act as a scaffold for elastic fiber deposition (28). Fibrillin-1 (Fbn1) and fibrillin-2 (Fbn2) are the main microfibril proteins (145). Fbn1 is clearly important for alveolarization and the structural homeostasis of the alveoli, since *Fbn1*$^{-/-}$ mice exhibited an alveolarization defect (146), and fibrillin fibers were fragmented and disorganized in emphysema (147). In addition to imparting structural properties to elastic fibers, fibrillins may also help to mediate elastic fiber assembly, such as lysyl oxidase cross-linking of elastin fibers (28), which is thought to be highly relevant to lung development (27, 64, 72, 89, 148, 149). Similarly, Fbn1 played a role in anchoring LTBP to ECM components (58). Changes in fibrillin expression have been noted in animal models of BPD. In mechanically ventilated mice, the ratio of Fbn1:Fbn2 was increased, with elevated Fbn1 expression and reduced Fbn2 expression noted (23, 99). By contrast, in the mouse hyperoxia model of BPD, the expression of both *Fbn1* and *Fbn2* mRNA was elevated (51). With these ideas in mind, disturbances to fibrillin expression may impact lung development either by directly modulating the physical properties of elastic fibers or by altering TGF-β dynamics in the ECM. These ideas await experimental investigation.

Tenascin C

Tenascins are a five-member family of large ECM glycoproteins, with tenascin C (Tnc), which is expressed in myofibroblasts, and endothelial, smooth muscle, and type II cells (81, 83), being the most studied in lung development (84) (**Table 1**). Tnc was comparatively highly expressed during human and animal development, including in the lung, particularly during the pseudoglandular and canalicular stages (84), at sites of active branching. Tnc is important for alveolarization, since *Tnc*$^{-/-}$ mice exhibited an alveolarization defect (150). Along these lines, administration of dexamethasone to developing mouse pups blunted alveolarization, which was accompanied by decreased Tnc expression (56), although the impact of dexamethasone on Tnc expression was not causally linked to the blunted alveolarization. In contrast to these findings, *TNC* expression was elevated in the lungs of patients

with BPD (83), which is consistent with the ability of TGF-β to drive *Tnc* expression in primary mouse fibroblasts *in vitro* (95). Tnc clearly plays a role in normal lung alveolarization; however, a causal role for changes on Tnc expression in aberrant lung alveolarization has yet to be demonstrated.

Fibronectin

Fibronectin (Fn1) is a large (440 kDa) glycoprotein dimer, consisting of two almost identical subunits. Fn1 has been reported both as a soluble form in plasma and as an insoluble form associated with the ECM, where Fn1 binds collagen (**Figure 2**), as well as Tnc, and other ECM components (151). Fn1 is expressed in the lung (152), in interstitial fibroblasts, endothelial cells, and smooth muscle cells, but not in epithelial cells (**Table 1**). Fn1 expression was highest during lung development, and very low in adult lung tissue (152). *Fn1*$^{-/-}$ mice exhibited early embryonic lethality (153), and a role for *Fn1* in lung development has not been demonstrated but is assumed. Several studies have documented the increased expression of Fn1 in clinical BPD, including in plasma, in endotracheal aspirates, and in BAL fluid (60, 104, 154, 155), as well as in lung tissue (60). This is consistent with the ability of TGF-β to drive Fb1 expression in lung fibroblasts (152). To date, no causal role for Fb1 in normal or aberrant lung development has been demonstrated. However, one exciting observation has suggested that decreased miR-206 expression in both clinical and experimental BPD may underlie the increased levels of *FB1* noted in the lungs of BPD patients (60, 105), since *FB1* has been described to be a target of miR-206 (105). Furthermore, miR-206 levels were decreased, whereas *Fb1* levels were increased in lungs from hyperoxia-exposed mouse pups (105). Taken together, these data make a compelling argument for the miR-206/Fb1 axis in aberrant alveolarization associated with BPD, although this idea requires experimental demonstration.

Fibulins and Emilins

Fibulins and emilins promote proper elastin fiber formation, by mediating protein–protein interactions between ECM proteins, or between the ECM and ECM remodeling enzymes, such as lysyl oxidases (156). Fibulins are small calcium-dependent glycoproteins that bind elastin (**Figure 2**). Fibulin-5 (Fbln5; also called developmental arteries and neural crest EGF-like protein, DANCE) has been reported to play a role in lung alveolarization. *Fbln5*$^{-/-}$ mice exhibited short, fragmented, and thickened elastin fibers, as well as a pronounced arrest of alveolarization (29, 157). No studies have examined a role for fibulins in clinical BPD; however, studies in animal models of BPD consistently revealed increased expression of Fbln5 in mouse pups exposed to hyperoxia (51, 72). Since TGF-β can drive Fbln5 expression (158), increased Fbln5 production after hyperoxia exposure may have been due to the attendant increased TGF-β signaling seen in this model (95). Changes in fibulin expression also appear to be sensitive to mechanical ventilation, where Martin Post's group demonstrated that *Fbln5* expression was impacted by the duration and intensity of tidal volume ventilation, and breathing frequency, when rats were mechanically ventilated with room air (103). Conversely, Richard Bland's group did not detect any impact of mechanical ventilation on Fbln5 expression when mice were ventilated with room air; however, ventilation with 40% O_2 reduced Fbln5 levels in the lung, which was accompanied by blunted alveolarization. While the *Fbln5*$^{-/-}$ mouse studies have implied a role for Fbln5 in alveolarization, the impact of increased Fbln5 expression on secondary septation and the development of the alveoli await

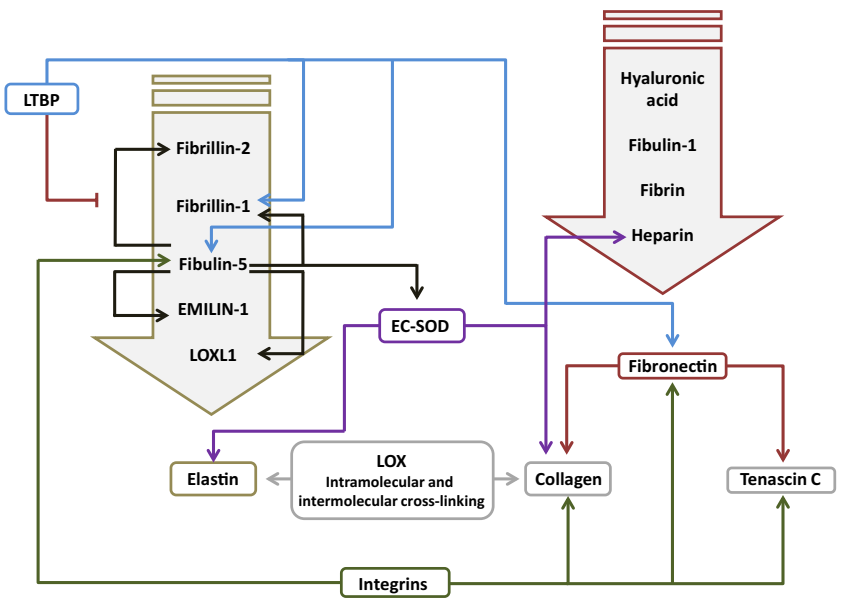

FIGURE 2 | Interactions between components of the extracellular matrix in the lung. The primary interacting molecules for elastin and fibronectin are collected together above the respective target molecules in the downward-pointing block arrows. Abbreviations: EC-SOD, extracellular superoxide dismutase; EMILIN-1, elastin microfibril interfacer 1; LOX, lysyl oxidase; LOXL1, lysyl oxidase-like 1; LTBP, latent transforming growth factor-β-binding protein.

demonstration. By way of speculation, Fbln5 promoted activation of MMP-2 and MMP-9 (159), which have also been associated with clinical and experimental BPD (see below). This, together with the possibility that Fbln5 over-expression might disturb elastic fiber formation, and might regulate the association of superoxide dismutase (SOD) (160) and lysyl oxidase-like 1 (LoxL1) (64) with the ECM, suggests avenues by which fibulin over-expression may influence lung development.

Like fibulins, emilins are a related group of elastic fiber-associated proteins (**Figure 2**), which impact elastogenesis, and have been reported to be expressed in the lung (161). Elastin microfibril interfacer 1 (Emilin-1) expression has not been studied in clinical BPD. However, Emilin1 expression has been reported to be dysregulated in animal models of BPD, including the hyperoxia exposure [*Emilin1* mRNA expression up-regulated; (23, 72)] and mechanical ventilation [Emilin1 protein expression down-regulated; (23)] in mice. *Emilin1*$^{-/-}$ knockout mice do exist (162), although no lung phenotype has been reported. However, the reported dramatic (2,000-fold) up-regulation of *Emilin1* expression in c-Jun N-terminal kinase (Jnk) knockout mice was reported to be accompanied by an alveolarization defect, perhaps implicating Emilin1 in the alveolarization process (163), although a dramatic up-regulation of *Fbln1*, *Fbln5*, and *Eln* expression was also noted in that study.

Latent TGF-β-Binding Proteins

The LTBP family consists of four extracellular MAGPs (164), which interact with, thereby modulate the activity of TGF-β. Ltbp1, Ltbp3, and Ltbp4 are reported to all associate with the small latent complex of TGF-β ligands and latency-associated propeptide (LAP), to generate the large latent complex (164). The LTBP family members are structurally related to fibrillins and were reported to interact with the ECM and play a role in ECM assembly. *Ltbp1*$^{-/-}$ mice exhibited perinatal lethality with heart defects, while a lung phenotype was not studied or reported (165). By contrast, both *Ltbp3*$^{-/-}$ and *Ltbp4*$^{-/-}$ mice exhibited an arrest of alveolarization (166) that was more pronounced in *Ltbp4*$^{-/-}$ mice (167). Ltbp4, which is known to bind Fbln5 (167), is believed to independently modulate elastogenesis and TGF-β activity, and thus, regulate lung development (168). The function of Ltbp2 remains elusive (164), but it has been suggested that Ltbp2 plays a TGF-β-independent role in elastogenesis (141), and Ltbp2 has been co-localized with fibronectin and Fbn1 in lung fibroblast cultures (58). Studies on Ltbp2 are complicated by the embryonic lethality reported in *Ltbp2*$^{-/-}$ mice (169). Interestingly, despite a clear role in alveolarization, no studies, to date, have examined the expression of LTBP family members in clinical or experimental BPD. These exciting studies await experimental investigation.

Polysaccharide Conjugates

Heparin, heparan sulfate, hyaluronic acid (hyaluronan), and chondroitin sulfate are polysaccharides or polysaccharide conjugates that have been reported to be mediators of lung alveolarization (170–172). Proteins carrying these conjugates, such as syndecan, which contains both heparan sulfate and chondroitin sulfate, exhibited molecular polymorphism – notably changes in the length of the heparin sulfate chains – over the course of lung development (173), implicating a role for heparan sulfate proteoglycans in lung development.

Temporal and spatial changes in glycosaminoglycan synthesis by lung fibroblasts have also been reported during lung development (174). Notably, fibroblasts in close proximity to the epithelium secreted hyaluronan, while more distant fibroblasts produced heparan sulfate and chondroitin sulfate during the pseudoglandular stage of lung development. During later stages of lung development, these fibroblasts switched to producing more hyaluronan, which was coincident with the thinning of the alveolar walls during the canalicular and later developmental stages. These authors postulated that developmentally regulated glycosaminoglycan generation by lung fibroblasts facilitated lung epithelial–mesenchymal interactions, which guided aspects of lung development (174).

Heparin and heparan sulfate have been reported to be the predominant glycosaminoglycans in epithelial basement membranes of the alveolus, and granules associated with collagen fibers of the basement membrane contained proteoglycan aggregates, which included chondroitin or dermatan sulfate (175). Heparan sulfate has been localized in the basement membrane during the embryonic, canalicular, and later phases of lung development (176). Heparan sulfate has received particular attention as a growth factor-binding protein, particularly in the context of fibroblast growth factor (FGF)-10, where Wellington Cardoso's group has provided evidence that FGF-10 induction of local budding during early lung development is directed by developmentally regulated regional patterns of heparan sulfate sulfation (177). This idea has also been extended to cytokines, such as interleukin (IL)-1 in the developing chick lung (178), as well as members of the bone morphogenetic protein (BMP) family (179).

Several studies in transgenic mice have highlighted causal roles for enzymes of the heparan sulfate biosynthetic pathway in lung development. For example, deletion of *N*-deacetylase/*N*-sulfotransferase (heparan glucosaminyl) 1 (Ndst1) led to pulmonary hyperplasia and acute respiratory distress in mice, possibly due to decreased surfactant production as a result of type II cells to mature (180). Similarly, deletion of glucuronyl C5-epimerase (Glce) in mice caused embryonic lethality, and stunted embryonic lung development, which was accompanied by a total loss of L-iduronic acid in heparan sulfate conjugates (181). Specifically concerning late lung development, defects in the development of the airspaces have been noted in both heparan sulfate 6-*O*-sulfotransferase 1 (Hs6st1) (182) and sulfatase 2 (Sulf2) (183) knockout mice. Apart from mice lacking enzymes involved in the heparan sulfate biosynthetic pathway, mice lacking heparan sulfate proteoglycans also exhibit lung development phenotypes. For example, deletion of glypican-3, a member of a family of heparan sulfate proteoglycans linked to the cell surface through a glycosyl-phosphatidylinositol anchor, generated abnormal lung structures in mice (184). These studies validated the earlier suggestion that heparin and heparan sulfate are mediators of lung development, although most work has been confined to the earlier stages of lung development that precede alveolarization. The generation of antibodies that detect specific heparan sulfate epitopes has facilitated the identification

of spatio-temporal changes in heparan sulfate structure during normal lung development, and aberrant lung development associated with congenital diaphragmatic hernia (CDH) (65, 185), which will facilitate further mechanistic work in this area. To date, exactly how these structural abnormalities to heparan sulfate proteoglycans results in disturbed alveolar structure remains to be clarified.

In addition to heparan sulfate proteoglycans, some work in embryonic lung explants has also revealed a role for chondroitin sulfate proteoglycans in early lung development (186). Furthermore, an interesting connection with inflammatory cells has been made, with the suggestion that CD44-positive macrophages, which take up hyaluronan, may regulate the steady-state levels of hyaluronan during lung development (187). Further work in this area should examine *how* defined alterations to proteoglycan structures direct proper development of the lung. The existence of transgenic mice and monoclonal antibodies that allow the specific detection of proteoglycan structures will facilitate these efforts. Additionally, no studies, to date, have examined changes in the expression of proteoglycan biosynthetic enzymes, or proteoglycan structures, in animal models of BPD.

ECM-INTERACTING MOLECULES

Integrins

Integrins are large heterodimeric transmembrane glycoproteins associated with various elements of the ECM. Integrin ligands include collagen I, Tnc, Fb1, laminins, TGF-β, and tissue transglutaminase (Tgm2), among many others. Each integrin dimer consists of a single α and β subunit. There are many integrin subunits, with 18 α and 8 β subunits having been identified in humans, to date (67). Expression of integrins is known to be dynamically regulated during lung development, where integrin-mediated cell–ECM interactions are known to play an important role (68, 188). Integrin expression has been noted during alveolarization, with the α2, α3, α6, and β1 subunits having been reported to be expressed in the bronchial and alveolar epithelium during the alveolar stage of lung development, as well as in adult lungs. By contrast, the α4 subunit was reported to be expressed in the respiratory epithelium only during lung development and has not been detected in adult lungs (68, 69, 188). The fibronectin receptor, integrin α8β1 (189) has been demonstrated to play a particularly noteworthy role in early and late lung development, where the Lawrence Prince's group demonstrated that *in utero* exposure of developing embryos to bacterial lipopolysaccharide (LPS) caused a reduction in expression of *Itga8*, which encodes the α8 integrin subunit, in mesenchymal cells (30). Thus, these authors examined lung structure in *Itga8*$^{-/-}$ mice, which exhibited a pronounced disturbance to the developing lung structure, including lobar fusion and alveolar simplification. Additionally, elastin fibers in these mouse lungs were described to be "wavy and short." This led these authors to suggest that integrin–ECM interactions played a notable role in late lung development. This idea is supported by observations made in the mouse hyperoxia BPD model, where increased expression of *Itgav*, encoding the α_v integrin subunit [which also binds fibronectin; (67)], was noted (51), and was accompanied by impaired alveolarization and increased *Fbln5* expression and TGF-β activity, and aberrant elastin fiber deposition. These studies have opened up an exciting new avenue, that is, the role of integrin-mediated ECM interactions in the regulation of alveolarization.

Extracellular Superoxide Dismutase

Extracellular superoxide dismutase (EC-SOD or Sod3), is one of three forms of SOD, a group of antioxidant enzymes representing the major cellular defense against the superoxide anion (O_2^-) (54, 190). EC-SOD is the only ECM-related antioxidant and has been reported to be the most abundant SOD in the lung (191). EC-SOD was reported to be expressed primarily in vessels, large airways, and alveolar septa. EC-SOD binds heparin (192) and heparan sulfate proteoglycans on the cell surface, and components of the ECM (54). EC-SOD binds collagen I (**Figure 2**) and is thought to protect against oxidative damage to collagen I (193). EC-SOD is also known to bind to tropoelastin, a process that is mediated by Fbln5 (160). EC-SOD is believed to play a role in protecting the ECM from oxidative damage, since reactive oxygen species (ROS) drive elastin degradation and increased collagen cross-linking (194, 195). Thus, EC-SOD might protect the developing and adult lung from oxidative damage (54), since EC-SOD was reported to be expressed throughout life (196), although EC-SOD protein expression and activity were blunted by hyperoxia exposure in adult mice (197). Along these lines, adult *Sod*$^{-/-}$ mice exhibited increased sensitivity to hyperoxic damage, with reduced survival and more pronounced alveolar edema, compared to wild-type mice; thus, supporting a role for EC-SOD in protection against oxidative damage to the lung (198). In support of this idea, over-expression of EC-SOD in transgenic neonatal mice protected against the damaging effects of hyperoxia on lung alveolarization (196), and expression of EC-SOD in a mouse lung epithelial cell-line protected against oxidative damage-induced cell death (55). The protective effects of EC-SOD over-expression on lung epithelial cells has also been demonstrated *in vivo* in hyperoxia-exposed newborn mice (199). None of these studies addressed collagen or elastin fiber integrity.

A wide spectrum of other ECM-interacting proteins still remains to be studied in the context of lung alveolarization. These proteins include the MAGP family members (142), as well as the small leucine-rich proteoglycans, such as decorin and related molecules, which play key roles in driving collagen fiber formation (200, 201). These future studies will no doubt add to the list of ECM-associated proteins that impact normal and aberrant late lung development.

ECM REMODELING ENZYMES

Matrix Metalloproteinases and Their Inhibitors

Matrix metalloproteinases are a large family of endopeptidases responsible for ECM breakdown and remodeling, which are necessary processes for proper formation of the ECM (9, 202). Different MMPs preferentially degrade different components of

the ECM, with MMP-1 and MMP-8 active against fibrillar collagens, and MMP-2 and MMP-9 preferentially active against basement membrane collagen (collagen IV), fibronectin, and elastin (77, 203–206). The proteolytic activity of MMPs can be regulated by MMP binding to cognate inhibitors, such as tissue inhibitor of metalloproteinases (TIMPs) (110, 207) (**Figure 3**). The expression of MMPs in the lung is known to be dynamically regulated over the course of lung development (**Figure 1**), with a progressive decrease in MMP-2 and MMP-14 [also called membrane-type-1 (MT1)-MMP] expression, but a progressive increase in MMP-9 expression between E10 and P21. These trends imply a role in lung alveolarization (9, 75, 78). Expression of MMP-2 and MMP-14 has been noted in airway and alveolar epithelial cells, endothelial cells, and fibroblasts (74–76), whereas MMP-9 was reported to be expressed in epithelial cells, fibroblasts, and inflammatory cells, including neutrophils and alveolar macrophages (61, 77, 79) (**Table 1**). MMP expression in the lung was driven by exposure of adult (77) and neonatal (93) rodents to hyperoxia. Similarly, elevated MMP expression has been noted in endotracheal aspirates or BAL fluid from preterm infants with BPD (109, 111–113). MMPs also played a role in alveolar destruction in experimental emphysema in mice (208). MMPs might impact alveolarization directly, through degradation of ECM components, or indirectly, through activation of growth factor pathways. For example, MMP-9 activated TGF-β signaling, which in turn stimulated lung fibroblasts to contract (61, 209). MMP-9 appeared to be able to influence lung alveolarization, since *Mmp9*$^{-/-}$ mice exhibited worsened lung development, in a mouse model where lung alveolarization was blocked by over-expression of IL-1β (210). In an alternative hyperoxia-based BPD model, *Mmp9*$^{-/-}$ mice were protected against the blunted alveolarization usually seen in the mouse hyperoxia BPD model (93). The reasons for these two discordant observations are currently unclear, however, may be related to the different models employed. Along the same lines, *Mmp14*$^{-/-}$ mice initially exhibited a 40% decrease in alveolar surface area compared to wild-type mice early during post-natal lung development (76), which was accompanied by thickened elastin fibers. By contrast, *Mmp2*$^{-/-}$ mice exhibited a "delayed" alveolarization, where an alveolarization defect was noted at P7, but alveolarization was normalized at P14 (76). It would be interesting to explore the impact of hyperoxia or mechanical ventilation of the *Mmp2*$^{-/-}$ and *Mmp14*$^{-/-}$ mice on alveolarization.

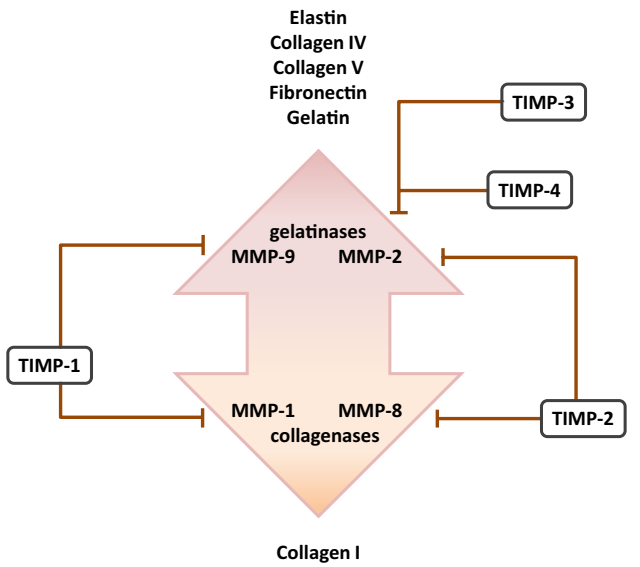

FIGURE 3 | Cognate inhibitors and target substrates of matrix metalloproteinases. The target substrates of the gelatinase and collagenase members of the matrix metalloproteinase (MMP) family are indicated, together with selected target MMP substrates. Abbreviation: TIMPs, tissue inhibitor of matrix metalloproteinases.

Several studies have addressed MMP expression in clinical BPD cases, where reduced MMP-2 levels were noted in endotracheal aspirates (109) and plasma (211), but increased MMP-8 levels were noted in endotracheal aspirates (111) and BAL fluid (112) from preterm infants with BPD. Increased MMP-9:TIMP-1 ratios have also been detected in BAL fluids from preterm infants that developed BPD (114) (**Figure 4**). Additionally, Ekekezie and coworkers (113) observed an increased MMP-9:TIMP-1 ratio in endotracheal aspirates from BPD patients, which correlated with poor patient outcome. These trends largely parallel observations made in animal models of BPD, where increased levels of MMP-2 and MMP-9 proteins were noted in hyperoxia-exposed mouse pups (93). Similarly, MMP-9 levels were modulated in the lungs of hyperoxia-exposed rats (78), and increased MMP-9 levels and an increased MMP-9:TIMP-1 ratio were noted in a premature baboon BPD model (107). Not all trends in MMP expression are consistent between investigations. For example, Hosford and co-workers reported the *decreased* expression of MMP-9 and *increased* expression of TIMP-1 in the rat hyperoxia model of BPD (110), which was also accompanied by blunted alveolarization. These discordant observations might be attributed to the extraordinary variation in the application of the BPD models: (i) newborn rats exposed to >90% O_2 for 9 days versus (ii) ventilated, premature baboons versus (iii) rats exposed to >95% O_2 between P4 and P14. Irrespectively, the general trend is toward increased MMP-9 activity in aberrantly developing lungs.

Lysyl Oxidases

Lysyl oxidases constitute a family of five members: the archetypical lysyl oxidase (Lox) and four lysyl oxidase-like enzymes (Loxl1–LoxL4) (212, 213). All lysyl oxidases catalyze the oxidative deamination of lysine and hydroxylysine residues, generating reactive semialdehydes, which then form intramolecular and intermolecular covalent cross-links in both elastin and collagen molecules (212, 213). Lysyl oxidases have been reported to play an essential role in normal lung development and have been implicated in the pathogenesis of several lung diseases, including pulmonary hypertension (73), lung adenocarcinoma (214), and BPD (72). Lysyl oxidases are known to play an important role in organogenesis, with *Lox*$^{-/-}$ mice exhibiting perinatal lethality, ostensibly due to a failure of the cardio-respiratory system (215, 216). Furthermore, Lox has been specifically implicated in the development of the respiratory system, where Lox has been reported to be required for the integrity of elastic and collagen fibers in multiple tissues (27). Interestingly, genetic ablation

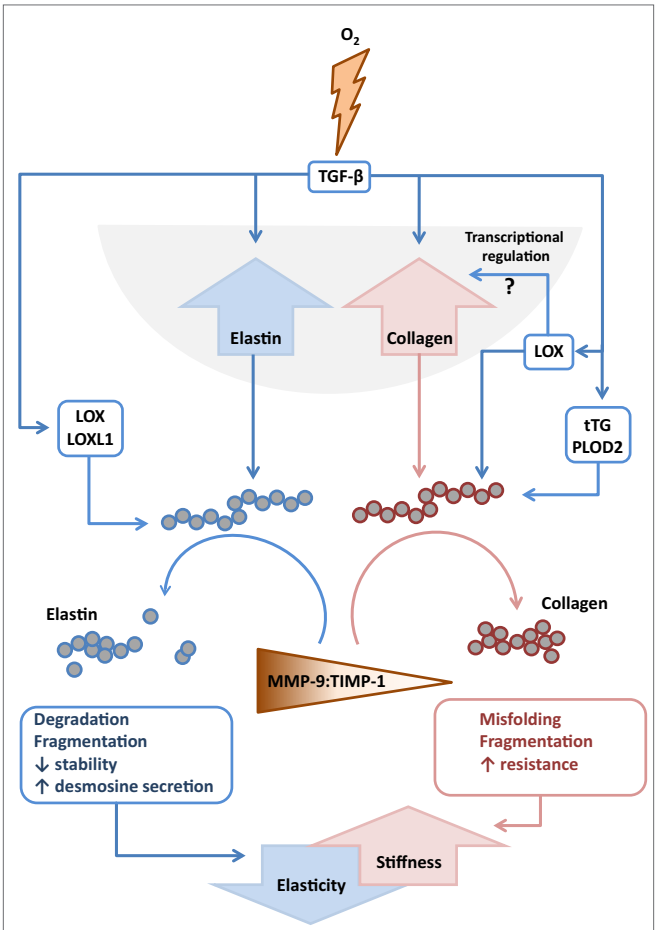

FIGURE 4 | Interactions between extracellular matrix components and remodeling enzymes driven by exposure to hyperoxia. The extracellular matrix remodeling processes that are described to be driven by exposure of the developing lung to hyperoxia that lead to increased stiffness and decreased elasticity of the developing lungs. Abbreviations: LOX, lysyl oxidase; LOXL1, lysyl oxidase-like 1; MMP, matrix metalloproteinase; TGF-β, transforming growth factor-β; TIMPs, tissue inhibitor of matrix metalloproteinases; tTG, tissue transglutaminase.

exclusively of Lox expression significantly reduced total lysyl oxidase activity, suggesting that Lox is the primary contributor out of the five family members, to lysyl oxidase-mediated effects. In *Lox*$^{-/-}$ mouse lungs, both desmosine and hydroxyproline levels were decreased relative to wild-type mice (216). While viable, *Loxl1*$^{-/-}$ mice exhibited connective tissue weakness, and developed pelvic organ prolapse and *cutis laxa* (64). Furthermore, *Loxl1*$^{-/-}$ mice exhibited alveolar simplification and reduced lung desmosine levels, implying a role in lung development, as well as perturbed elastin fiber structures throughout the organism (64).

Lysyl oxidase expression has been studied in animal models of BPD. Increased Lox and Loxl1 expression has been noted in the lungs of preterm ventilated lambs (24). Lox expression was also increased by mechanical ventilation in mouse pups (23), but lung Loxl1 levels were reduced in mechanically ventilated mice (23). Elevated lysyl oxidase activity (72, 89, 94), and elevated Lox and Loxl1 levels (72, 89) were detected in newborn mice exposed to normobaric hyperoxia. This has led some investigators to propose that the ECM in affected lungs might be "over cross-linked," and thus excessively stabilized, which has been proposed to be a potential contributing factor in arrested alveologenesis associated with clinical and experimental BPD (72, 89). Consistent with elevated Lox expression and activity, exposure to hyperoxia also generated increased amounts of insoluble collagen and the dihydroxylysinonorleucine (DHLNL) collagen cross-link, as well as an increased DHLNL:hydroxylysinonorleucine (HLNL) ratio, and disordered elastin organization in the alveolar septa (89). To address a causal role for lysyl oxidases in blunted alveolarization in the hyperoxia BPD model, newborn mice were treated with the pan-lysyl oxidase inhibitor β-aminopropionitrile (BAPN), which did not improve lung alveolarization, but did improve elastin organization assessed by visual inspection (89). This might indicate that the partial normalization of elastin organization in developing septa alone (through normalization of lysyl oxidase activity) was not sufficient to normalize lung alveolarization in the mouse hyperoxia BPD model. Several questions regarding lysyl oxidases and alveolarization come to mind, among them: what role do the different lysyl oxidases play in lung development, and in which tissues? Lysyl oxidases are expressed in several different cell types (**Table 1**), and it may be that different lysyl oxidases have different contributions to lung alveolarization, acting in different cell types. The generation of conditional, inducible deletions of the various lysyl oxidase genes would help to address this question. Additionally, "non-matrix" roles for lysyl oxidases should also be considered, where lysyl oxidases have been reported to modulate gene regulation in the nucleus, for example, the expression of *COL3A1* (217). This has revealed nuclear functions – primarily of LoxL2 – which modulated epigenetic effects in the nucleus by deamination of trimethylated Lys^{4} in histone H3, which was linked to transcriptional repression (218). Furthermore, LoxL2 regulated keratinocyte differentiation independent of lysyl oxidase catalytic activity (219). Similar studies have yet to be performed with other lysyl oxidases, but highlight possible roles for lysyl oxidases in lung alveolarization that are not related to ECM cross-linking.

Lysyl Hydroxylases

The ability of lysyl oxidases to generate covalent cross-links requires lysine or hydroxylysine residues in ECM substrates. These hydroxylysine residues are generated by another family of enzymes, the lysyl hydroxylases (officially named procollagen-lysine, 2-oxoglutarate 5-dioxygenases, or PLODs) (220), which consist of three family members: PLOD1–PLOD3. A role for lysyl hydroxylases in organ development was underscored by the early embryonic lethality of *Plod3*$^{-/-}$ mice (221), while *Plod1*$^{-/-}$ mice were viable, but exhibited vascular pathology and abnormal collagen fiber structure (222). This family of ECM-modifying enzymes is relatively poorly characterized. Some evidence does exist illustrating that lysyl hydroxylases play a role in aberrant late lung development, both in humans and in mice. A recent study by Witsch and colleagues (80) revealed that the lung expression of PLOD family member PLOD2 was up-regulated in premature infants with BPD. Furthermore, *Plod1*, *Plod2*, and *Plod3* expression was elevated in the lungs of mice in the hyperoxia BPD model

(80), and the elevated *Plod2* expression was mediated by TGF-β. These data indicate that the lysyl hydroxylases may play a role in normal and abnormal lung development, and this possibility awaits experimental attention.

Transglutaminases

The transglutaminases constitute an eight-member family of calcium-dependent enzymes, which cross-link collagens and fibronectin, among other proteins (223). Of the transglutaminases, largely transglutaminase 2 (Tgm2; also called tissue transglutaminase, tTG) has been studied in lung disease and was reported to be expressed in fibroblasts, as well as epithelial, endothelial, and smooth muscle cells (85, 224). In addition to cross-linking activity, Tgm2 is an integrin-binding adhesion co-receptor for fibronectin (225). Tgm2 has also been implicated in lung fibrosis (86, 226, 227), allergy (87), cystic fibrosis (228, 229), and pulmonary hypertension (230). Tgm2 has further been credited with a role in organogenesis (231), including lung development (232). In preterm infants with BPD, *TGM2* mRNA levels were elevated (85), which was also seen in the lungs of hyperoxia-exposed newborn mice with experimental BPD (85). In the case of hyperoxia-exposed newborn mice, increased *Tgm2* levels were driven by TGF-β, most likely in lung epithelial cells. This is particularly noteworthy because not only can TGF-β drive Tgm2 expression but Tgm2 can also activate TGF-β (233), suggesting a possible vicious circle of Tgm2 expression and TGF-β activation in aberrant lung alveolarization. These studies indicate that changes in transglutaminase expression are associated with normal and aberrant alveolarization; however, a causal role for transglutaminases in lung development has yet to be experimentally documented. The existing *Tmg2*$^{-/-}$ knockout mice would be an ideal starting point for these studies (234).

PERSPECTIVE

Given that, the ECM plays a pivotal role in lung development, it comes as no surprise that perturbations to ECM production and remodeling accompany defective secondary septation and aberrant alveolarization associated with BPD. Identification of the perturbations to ECM organization that play a causal role in aberrant alveolarization would assist in our understanding of the pathological processes that disturb late lung development. Equally important is the delineation of pathogenic pathways that drive these causal disturbances to ECM structure.

Roles for ECM structural proteins and ECM remodeling enzymes in lung alveolarization have been identified using gene knockout approaches. These studies have provided a very solid foundation for future work but are complicated by the pre- or peri-natal lethal phenotype of some knockout mice. This has been partially remedied by the parallel over-expression of human genes, or genes with altered promoter activity, in the background of a homozygous-null strain (such as the expression of the human *ELN* gene in *Eln*$^{-/-}$ mice, described above), which overcome the lethality of the homozygous-null mutants, and facilitated further studies on the gene products of interest. However, this has been more the exception than the norm. Additionally, it is widely recognized that the discrete expression of particular *genes*, in particular, *cell types* at particular *stages* of lung development is the basis of the highly coordinated program of the generation of a very complex organ (1, 2). This makes the use of constitutive global knockout mouse strains problematic.

Rapidly evolving mouse transgenic technology makes an increasing number of conditional-ready gene-deletion strains available through the use of floxed alleles. In combination with inducible Cre-recombinase systems, these conditional strains become inducible, conditional strains, which facilitate gene deletion in developing mouse pups at particular time points during post-natal lung development, in restricted cell types. These approaches rely largely on the use of doxycycline-inducible rtTA $(tetO)_7$-Cre and tamoxifen-inducible Cre^{ERT2} systems. These inducible, conditional-ready mouse strains will prove invaluable in assessing how the temporal and tissue-specific expression of particular genes during lung development impacts lung development *per se* (235). Among the drawbacks of this approach are the limitations of some floxed allele strains, which would have to be created *de novo*, and also, the lack of – or technical difficulties with the use of – some driver lines. For example, no suitable driver line currently exists that can exclusively target lung fibroblasts, or that can discriminate between airway and vascular smooth muscle cells (235). Remaining with transgenic mice, most studies, to date, have evaluated the loss of a particular gene on lung development. However, particularly in the context of animal models of BPD, genes might be over-expressed or up-regulated, rather than down-regulated. As such, to be able to "phenocopy" a lung phenotype by over-expressing a gene of interest, *in the correct cell-type at the correct time*, would go a long way to validate candidate pathogenic mediators of arrested alveolarization. Along these lines, many knockout and pharmacological intervention studies have identified new "players" in normal lung alveolarization (such as LTBP and elastin and collagen cross-linking enzymes), but a contribution to pathological lung development in animal models of BPD has not been undertaken. These exciting studies may well reveal new pathogenic pathways that drive aberrant lung alveolarization.

While elastin has received a tremendous amount of attention as a regulator of lung development, the collagens remained largely neglected. Since many candidate pathogenic mediators (such as elastin cross-linking enzymes) also influence collagen structure and function, it would be interesting to explore roles for disturbed collagen organization during lung development. Along these lines, the ratio of elastin:collagen is also noteworthy. In the hyperoxia models of BPD, there is a reported shift toward an increased collagen:elastin ratio. This would impact lung rigidity and elasticity, and thus lung development, which is dependent on physical forces generated by, for example, breathing motions.

Animal models of BPD have proved very important for the identification of candidate pathogenic mediators of normal and aberrant late lung development. These studies are often not followed up with validation studies that pin-point a role (*if any*) for a particular candidate mediator that exhibited changes in gene or protein expression in a BPD model. These studies are important, since changes in the gene or protein expression of a particular molecule may be (i) epiphenomenal (i.e., that the molecule in

question was a bystander without any role in the alveolarization process), (ii) causal (i.e., that molecule in question was a mediator of arrested alveolarization), or (iii) reparative (i.e., that molecule in question mediated a lung defense or repair program that was engaged during aberrant alveolarization, which aimed to restore proper alveolarization). It is very important to determine which of these three categories a "candidate" mediator of aberrant lung development falls into.

Interestingly, in many studies, once a candidate mediator of aberrant lung alveolarization was identified in an animal model of BPD, much effort was then expended on identifying how the candidate mediator impacted ECM structures during alveolarization. Rather, less energy is usually invested in understanding how the expression of the candidate mediator was altered by the injurious stimulus (for example, inflammation, hyperoxia, or mechanical ventilation). The identification of such proximal pathways would be important in a translational sense, where addressing the very proximal causes of arrested lung development might be therapeutically targeted, ultimately in affected patients. With this in mind, physical forces and oxidative stress might be good starting points to understand the activation of pathways that produce or remodel the ECM. Furthermore, ECM structures have recently been credited with a role in driving pluripotent cell differentiation in acellular lung scaffolds (236). Thus, how the ECM may shape stem cell niches in the developing lung, and direct phenotypic transformation of the constituent cell types of the developing lung are further areas that will no doubt receive attention in the coming years.

With the rapidly expanding repertoire of genetic tools, and the development of state-of-the-art methodology to study both lung alveolar architecture and the biochemical nature of the ECM, we have never been better positioned to explore the complex interactions of the ECM during lung alveolarization. It is clear that there is much exciting work to be done!

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REVIEW
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Aberrant Pulmonary Vascular Growth and Remodeling in Bronchopulmonary Dysplasia

*Cristina M. Alvira**

Department of Pediatrics, Division of Critical Care Medicine, Stanford University School of Medicine, Stanford, CA, USA

In contrast to many other organs, a significant portion of lung development occurs after birth during alveolarization, thus rendering the lung highly susceptible to injuries that may disrupt this developmental process. Premature birth heightens this susceptibility, with many premature infants developing the chronic lung disease, bronchopulmonary dysplasia (BPD), a disease characterized by arrested alveolarization. Over the past decade, tremendous progress has been made in the elucidation of mechanisms that promote postnatal lung development, including extensive data suggesting that impaired pulmonary angiogenesis contributes to the pathogenesis of BPD. Moreover, in addition to impaired vascular growth, patients with BPD also frequently demonstrate alterations in pulmonary vascular remodeling and tone, increasing the risk for persistent hypoxemia and the development of pulmonary hypertension. In this review, an overview of normal lung development will be presented, and the pathologic features of arrested development observed in BPD will be described, with a specific emphasis on the pulmonary vascular abnormalities. Key pathways that promote normal pulmonary vascular development will be reviewed, and the experimental and clinical evidence demonstrating alterations of these essential pathways in BPD summarized.

Keywords: pulmonary angiogenesis, pulmonary hypertension, alveolarization, chronic lung disease, VEGF, HIF, nitric oxide

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***Correspondence:**
Cristina M. Alvira
calvira@stanford.edu

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INTRODUCTION

A significant portion of lung development occurs after birth during the alveolar stage of development. During this final stage, the alveolar ducts divide into alveolar sacs by secondary septation, and the pulmonary capillary bed expands *via* angiogenesis to markedly increase the gas exchange surface area of the lung (1). However, postnatal completion of growth renders the lung highly susceptible to insults that disrupt this developmental program. This is particularly evident in the setting of preterm birth, where disruption of alveolarization causes bronchopulmonary dysplasia (BPD), the most common complication of prematurity (2). While advances in the supportive care of extremely premature infants have reduced mortality, the morbidities associated with severe BPD persist (3). Accompanying this increase in survival, the clinical and pathologic features of BPD have changed significantly. In contrast to the severe lung injury characterizing "old BPD" as originally described by Northway (4), premature birth earlier in gestation appears to disrupt the normal program of alveolar and vascular development, resulting in the "new BPD," characterized by an arrest in alveolar and vascular development (5).

The impaired pulmonary angiogenesis observed in patients with BPD appears to be the key to the pathogenesis. Proangiogenic factors are decreased in the lungs of infants dying from BPD (6) and in animal models of BPD induced by hyperoxia (7). Administration of anti-angiogenic agents to neonatal rats impairs both pulmonary angiogenesis and alveolarization (8, 9), and overexpression of proangiogenic factors, such as vascular endothelial growth factor (VEGF), rescues the adverse effects of hyperoxia on alveolarization (7). Moreover, in addition to simple decreases in pulmonary microvascular growth, the pulmonary vascular abnormalities in BPD may also include pathologic remodeling and heightened tone, leading to the development of pulmonary hypertension (PH), as well as an increase in the development of abnormal aorto–pulmonary communications, potentially promoting intrapulmonary shunting.

This review presents an overview of lung development and details the pathology of the "new" BPD, characterized by an arrest in normal lung development. Specific focus will be centered upon the pulmonary vascular abnormalities in BPD including impaired pulmonary angiogenesis, abnormal pulmonary vascular remodeling, heightened pulmonary vascular tone, and development of abnormal collateral circulations. Key pathways that promote normal pulmonary vascular development will be reviewed, and the experimental and clinical evidence demonstrating how these pathways are altered in BPD summarized.

OVERVIEW OF NORMAL AIRWAY AND PULMONARY VASCULAR DEVELOPMENT

Lung development begins when the primitive lung bud emerges from the ventral foregut and divides during the embryonic stage of development (4–7 weeks gestation), forming two lung buds lying on either side of the future esophagus and surrounded by splanchnic mesenchyme (10). The remaining four stages follow sequentially, beginning with the development of the pre-acinar airways *via* branching morphogenesis during the pseudoglandular stage (7–17 weeks gestation). During the canalicular stage (17–25 weeks gestation), the airways divide further to form the alveolar ducts, and the distal lung mesenchyme thins to allow close approximation of the developing respiratory epithelium and vascular endothelium. Widening and branching of these distal air sacs occurs in the saccular stage (26–36 weeks gestation), and finally, during the alveolar stage (36 weeks gestation onward), the terminal alveoli form by the process of secondary septation and rapidly increase in number throughout early childhood (11).

The mature lung contains approximately 500 million alveoli (12), each surrounded by a network of pulmonary capillaries allowing close proximity of the air filled alveolus with the blood-filled capillary. This intimate association of the pulmonary microcirculation with the terminal airspaces is imperative for efficient gas exchange. Therefore, the pulmonary blood supply must develop in close relationship to the airways throughout lung development (10). Early recognition that the branching of the pre-acinar arteries (formed by the end of the pseudoglandular stage) occurs at the same time and along a similar pattern, as the branching of the airways, suggested that the airways may provide a template for the development of the pulmonary arteries and veins (13).

The pulmonary circulation likely forms through a combination of vasculogenesis, the *de novo* formation of vessels from the differentiation of primitive angioblasts and hemangioblasts, and angiogenesis, the sprouting and branching of new vessels from existing vessels (14, 15). However, the degree to which each process contributes to the formation of the pulmonary vasculature at each stage of development remains a source for debate. Early evidence supported the notion that the proximal arteries form by angiogenic sprouting from the main pulmonary trunk and that distal branches form *de novo* in the distal mesenchyme *via* vasculogenesis. Using a method to make a cast of the developing pulmonary vasculature in fetal rats (from E9 to E20), deMelo et al. showed that isolated "blood lakes" form in the periphery of the lung (presumably by vasculogenesis) as early as E9. This was followed by the central sprouting of the proximal arteries, with the formation of five to seven generations of branching by E14, and connections between the proximal and distal vessels by E13–14 (16). In contrast, using transgenic reporter mice that express LacZ under the control of an endothelial specific promoter, Schachtner et al. found evidence of connections between the proximal, branching pulmonary arteries, and endothelial cells located in the distal mesenchyme as early as E10.5, several days before patency of the central pulmonary arteries has occurred. These findings suggested the authors that vasculogenesis may contribute to the development of the proximal pulmonary vasculature as well (17).

Prior to term birth, the density of the peripheral pulmonary vessels markedly increases in density, suggesting expansion of the capillary network by angiogenesis (16). After birth, the pulmonary capillary network continues to expand, resulting in a 35-fold increase by adulthood (13). Airway and vascular development are closely linked, with the disruption of one process impairing the other, and each culminating in a global disruption of lung development (18). Moreover, pulmonary vascular development continues throughout all stages of lung development in a manner proportional to the overall growth of the lung, rendering it vulnerable to perturbations occurring in both embryonic and postnatal life (17).

EXTREME LUNG IMMATURITY AND ARRESTED LUNG DEVELOPMENT: THE "NEW" BPD

In 1967, Northway et al. used the term BPD to describe a novel form of chronic lung disease that developed in preterm infants (mean gestational age of 32 weeks) who had a history of neonatal respiratory distress (4). This original form of BPD was associated with positive-pressure ventilation and prolonged oxygen therapy, and characterized by histologic evidence of severe lung injury (e.g., inflammation, protein-rich edema, airway epithelial metaplasia, and peribronchial fibrosis) and marked airway and pulmonary vascular smooth muscle hypertrophy (19, 20). Abnormalities in the pulmonary vasculature were also a feature of the disease. Pathologic examination of post-mortem lung tissue from a small

group of infants with BPD who survived for at least 1 month demonstrated decreased density of peripheral pulmonary arteries as compared to control patients, both by barium angiogram and histologic measures (21).

However, advances in medical therapy, including antenatal steroids, surfactant replacement therapy, and the institution of lung protective strategies of ventilation, have permitted the survival of extremely immature, very low birth weight (VLBW) infants. Accompanying this increase in survival, the clinical, radiographic, and pathological features of BPD have changed significantly. In contradistinction to the original form of BPD, birth of VLBW infants during the late canalicular or early saccular stages of lung development appears to disrupt the normal alveolar and vascular development, resulting in the "new BPD." Margraf et al. described the lung pathology of this new, post-surfactant form of BPD in a small case series of infants who died with severe BPD. One of the most striking findings observed by the authors was the severely reduced alveolar number in the infants with BPD compared to controls, with little evidence of the normal, physiologic increases in alveolar number typically observed with advancing age (22). Similarly, Husain et al. also showed evidence of arrested acinar development in a series of infants with post-surfactant BPD, including both reductions in acinar number and increases in acinar size (23).

Pathologic data obtained from autopsy specimens can be difficult to interpret and generalize to the entire disease population, as these samples often represent the most severe lung disease in patients with BPD (24). This is particularly true now that key advances in the medical care of preterm infants have markedly decreased mortality, such that infants who die from BPD in this era truly represent an extremely ill subset of patients. However, Coalson et al. obtained important information surrounding the evolving histopathology in infants with this "new" form of BPD in a small series that examined open lung biopsies from low-birth weight babies on ventilator support who received surfactant but not steroids. Those infants also demonstrated alveolar simplification but minimal metaplasia, and variable degrees of inflammation and abnormal extracellular matrix deposition (25).

ABNORMALITIES IN PULMONARY VASCULAR DEVELOPMENT AND REMODELING

Dysmorphic Pulmonary Microvascular Development

In addition to alveolar simplification (i.e., decreased complexity of distal lung septation), the pathology of this "new" form of BPD also appears to include abnormalities in the development of the pulmonary microvasculature. A comparison of autopsy specimens taken from infants dying from BPD compared to infants dying without lung disease at similar post-conceptional ages demonstrated that the lungs of infants with BPD had an overall reduction in immunostaining for the endothelial specific marker CD31, suggesting a decrease in pulmonary microvascular density. Moreover, the pulmonary capillaries, when present, appeared to be abnormally dilated and frequently located within thickened alveolar septa, rather than immediately adjacent to the alveolar epithelium (6). These reductions in the growth of the distal pulmonary vasculature were in keeping with the pathologic findings observed in specimens obtained from patients dying of BPD in the pre-surfactant era, where decreases in arterial number and cross-sectional area were thought to contribute to the increased dead space ventilation observed in those infants (26).

However, additional studies have suggested that rather than a simple decrease in pulmonary vascular growth, the vascular abnormalities observed in patients with BPD might be more accurately described as "dysmorphic." In the open lung biopsy samples obtained by Coalson et al., evidence of abnormal capillary development was apparent, with CD31 immunostaining demonstrating an "adaptive dysmorphic pattern of vascular organization." This pattern included a paucity of capillaries within the walls of the thinned abnormally enlarged alveoli, and dilated, more abundant capillaries in other sites (25). In contrast, a stereology-based assessment of endothelial cell volume in short- and long-term ventilated preterm infants demonstrated that total endothelial cell volume increased in ventilated infants as compared to age-matched controls, in association with an increase in total parenchymal volume, suggesting an expansion of the pulmonary microvasculature. However, in the long-term ventilated patients, the capillary network was simplified, had decreased branching, and retained the dual capillary pattern characteristic of the saccular lung, features predicted to decrease gas exchange efficiency (27). Taken together, these studies suggest that variable abnormalities in the pulmonary capillaries may be observed in BPD, with suppressed vascular growth at some stages of the disease, and excessive, dysmorphic growth at other stages, perhaps representing a maladaptive compensatory response.

Abnormal Muscularization, Heightened Vascular Tone, and the Development of Pulmonary Hypertension

In his original description of BPD, Northway noted that some patients had evidence of medial hypertrophy of the pulmonary arteries, suggesting the development of PH (4). This histologic finding was confirmed by clinical studies demonstrating elevations in pulmonary arterial pressures (PAPs) and pulmonary vascular resistance (PVR) by either cardiac catheterization or enchocardiography in survivors of BPD. In one such study, Fouron et al. found that the majority of patients with BPD in the "acute phase" had echocardiographic evidence of PH, and that pulmonary pressures remained high in those infants who eventually died, but normalized in infants who recovered (28). However, long-term follow-up of patients with pre-surfactant BPD and PH showed that in many patients, elevations in PAPs persisted through early childhood (29).

With the evolution of BPD in the post-surfactant era, the development of PH remains a significant feature of the disease for a subgroup of patients and significantly impacts long-term prognosis. In a prospective study of preterm infants using a broad echocardiogram-based definition of PH, early evidence of PH was found in more than 40% of patients at 7 days of age, and late PH found in almost 15% of patients at 36 weeks PMA. In patients

who develop severe BPD, the incidence of late PH appears to be significantly higher ranging from 30 to 50% of patients (30–32). Moreover, the presence of PH in patients with BPD is independently associated with a greater increase in the odds of death (30, 32), with mortality rates as high as 40% in some studies (33). Of note, the risk of death appears to be highest in the first 6 months after the diagnosis of PH, and the majority of infants with BPD and PH who survive beyond a mean of 10 months of age demonstrate an improvement in the severity of PH (33). Numerous risk factors have been associated with an increased incidence of PH in patients with BPD including: oligohydramnios (32, 34), low apgar scores (32, 34), postnatal sepsis (34), small for gestational age (33), and prolonged use of positive-pressure ventilation (31). Of note, while the risk of developing PH is significantly higher in patients with severe versus moderate BPD (32), a smaller percentage of infants with no, mild, or moderate BPD also develop late PH. This suggests that the risk for developing late PH may not be primarily dictated by the severity of lung disease (31). In addition, early PH appears to predict the development of BPD (35), again highlighting the link between abnormalities in the pulmonary circulation and impairments in distal lung development (**Figure 1**). While a complete understanding of the mechanisms leading to PH in a subset of patients is lacking, the data suggest that patients with BPD and PH demonstrate abnormalities in both distal pulmonary artery muscularization and tone.

Abnormalities in Pulmonary Arterial Muscularization

In his original report, Northway et al. identified "early vascular lesions of the pulmonary hypertensive type" in the cohort of infants in the later stages of the disease, which comprised medial hypertrophy and characteristic breakdown of the elastic lamina (4). Later studies demonstrated similar pathologic remodeling of small pulmonary arteries in patients with the pre-surfactant form of BPD. In a small study of preterm infants with severe BPD and cor pulmonale, affected patients demonstrated an increase in the percent medial thickness of distal arteries and an extension of arterial smooth muscle into peripheral arteries such that the majority of alveolar wall arteries were completely muscularized (26). Further, abnormal muscularization of the pulmonary arteries was often a feature of pre-surfactant BPD even in patients that did not develop cor pulmonale. In premature infants with respiratory distress syndrome (RDS) who died early in life while still requiring mechanical support, many demonstrated increased medial thickness of distal arteries, appearing similar to the muscularized small arteries characteristic of a term infant on the first day of life (20). Moreover, in keeping with the findings of Bush et al., those infants with BPD who developed cor pulmonale had evidence of marked muscularization of small arteries, with complete muscularization of arteriolar wall arteries, and some patients with intimal proliferation of larger arteries (20). In a separate study, the combination of abnormal muscularization

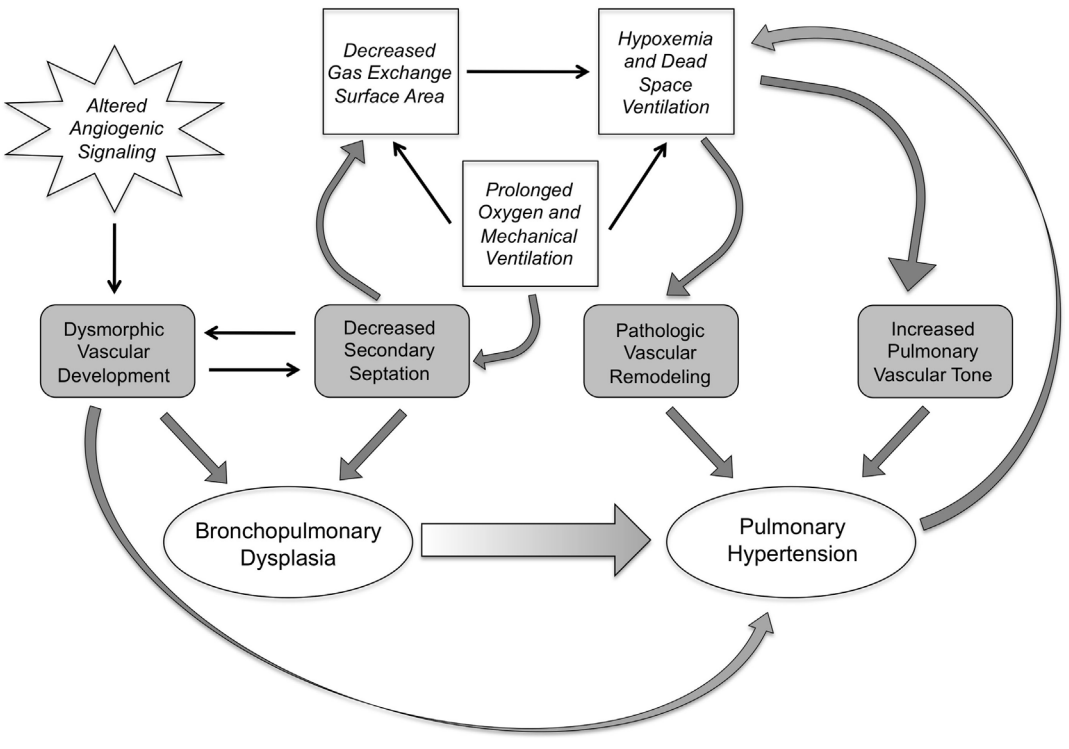

FIGURE 1 | The interplay of pathologic and clinical factors that lead to pulmonary hypertension in bronchopulmonary dysplasia. Dysmorphic vascular development as a result of altered angiogenic signaling combines with impairments in secondary septation, leading to the development of BPD. These events set the stage, pre- and postnatally, for the development of pulmonary hypertension. The decrease in gas exchange surface area resulting from the impaired secondary septation also sets up a vicious cycle of hypoxemia and dead space ventilation that prolongs the need for mechanical ventilation and oxygen therapy, and induces pathologic changes in pulmonary vascular remodeling and tone, further increasing the risk of pulmonary hypertension.

of distal arteries with variable degrees of either increased or decreased pulmonary capillary density suggested a "dual process of adaptation and response to injury in a hypoplastic lung" (36).

In the post-surfactant era, mortality of premature infants has decreased, thus limiting the availability of autopsy specimens that would allow careful characterization of the pulmonary arterial histopathologic changes in the "new" form of BPD. However, in at least one study, it appears that the abnormal muscularization of peripheral arteries remains a consistent pathologic feature. In a study of post-mortem tissue obtained from surfactant-treated preterm infants with BPD, there was evidence of increased arterial wall thickness and muscularization of distal vessels in preterm infants with severe BPD, although these histologic changes were less marked than those observed in specimens obtained from infants who developed PH in the setting of persistent pulmonary hypertension of the newborn (PPHN) and premature rupture of membranes (PROM) (37). While the mechanisms that specifically induce pathologic pulmonary vascular remodeling in BPD are unknown, they are hypothesized to include some of the well recognized injurious stimuli that disrupt distal lung growth including hyperoxia, mechanical ventilation, and inflammation (38).

Abnormalities in Pulmonary Vascular Tone

In addition to abnormal pulmonary arterial remodeling, heightened pulmonary arterial tone also appeared to be an important component of the PH observed in patients with pre-surfactant BPD. Survivors of BPD with persistent oxygen requirements and evidence of right ventricular hypertrophy (RVH) on ECG had evidence of PH on cardiac catheterization, with pulmonary vascular beds that were responsive to even low levels of oxygen (39). In a prospective study of 15 patients with moderate to severe BPD and PH undergoing cardiac catheterization, all patients demonstrated a reduction in PA pressure with supplemental oxygen, and variable responses to vasodilator therapy depending on the presence or absence of systemic–pulmonary collaterals (40).

Elevated pulmonary vascular tone remains a key feature of the PH in BPD survivors in the post-surfactant era. A study examining BPD survivors with PH who underwent cardiac catheterization found that most patients have significant pulmonary vascular reactivity, demonstrating elevations in mean PAP with hypoxia, and conversely, decreased mean PAP with the combination of hyperoxia and inhaled nitric oxide (iNO) (41). Similarly, in a study reporting data from the cardiac catheterization of 13 patients with BPD and PH, PAP and PVR decreased significantly with vasodilator therapy (100% O_2 or iNO) in the majority of patients, but still remained elevated above normal levels (33).

Abnormal Collateral Circulations

In an early report, cardiac catheterization of two premature infants who required prolonged mechanical ventilation found that although these infants had normal pulmonary pressures, they both had evidence of large systemic collaterals with left to right shunts, a finding the authors hypothesized likely contributed to their persistent ventilator dependence (42). This report was followed by the description of similar collateral vessels in a subgroup of patients with severe BPD and PH, in whom the administration of vasodilators had deleterious results, inducing respiratory acidosis, pulmonary edema, and more severe hypoxemia (40). However, it was not clear from either report whether these abnormal vessels were congenital in nature or acquired, resulting from persistent hypoxemia, pathologic alterations in pulmonary blood flow, or disrupted lung development (43).

More recently, histologic examination of lung tissue from a number of patients dying with severe BPD demonstrated the presence of numerous smaller intrapulmonary arteriovenous anastomotic vessels (IAAV) that appear similar to the "misalignment of veins" seen in alveolar capillary dysplasia. These vascular channels are located in the lobar periphery and extend toward the pulmonary arteries, appearing to connect with the microvascular plexus surrounding the pulmonary arteries and airways (44). Of note, these intrapulmonary anastomotic vessels are not unique to BPD, but observed in other diseases of impaired alveolarization. For example, in a similar study examining the lung tissue of infants dying from severe congenital diaphragmatic hernia (CDH) and associated PH, the lungs of all patients demonstrated prominent, engorged intrapulmonary vessels connecting the pulmonary veins to the microvessels surrounding the pulmonary arteries (45). Similar, intrapulmonary, bronchopulmonary anastomoses have also been noted in infants dying from meconium aspiration syndrome (46). These prominent IAAV may represent the failure of the normal fetal IAAV circulation to close after birth and have the potential to permit right to left intrapulmonary shunting, thus contributing to the hypoxemia observed in patients with severe BPD.

KEY PATHWAYS DIRECTING NORMAL PULMONARY VASCULAR DEVELOPMENT AND FUNCTION

Extensive clinical evidence obtained from patients with BPD in both the pre- and post-surfactant era has identified impaired and dysmorphic pulmonary vascular development as a key feature of the disease. These data suggest that the normal pathways that promote postnatal pulmonary vascular growth are disrupted in BPD. In this section, a number of key pathways that direct normal pulmonary vascular growth and function will be reviewed, and evidence demonstrating alterations in these pathways in experimental models of BPD (**Table 1**) and clinical studies will be summarized.

Vascular Endothelial Growth Factor

The endothelial cell mitogen and survival factor, VEGF, is essential for normal blood vessel development. Alternate splicing from a single gene produces three distinct isoforms: $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$. These three isoforms demonstrate differential binding to heparin sulfate and affinities for the two predominant receptors: tyrosine kinases fms-like-tyrosine kinase-1 (FLT-1) and fetal liver kinase-1 (FLK-1) (47). The lung expression of the two heparin-binding isoforms, $VEGF_{164}$ and $VEGF_{188}$, increases during the late saccular stage of development in the mouse and remains high through adulthood (47), with $VEGF_{188}$ becoming

TABLE 1 | Molecular mechanisms contributing to impaired alveolar and pulmonary vascular growth in animal models.

Molecule	Physiologic functions and disruption in animal models of BPD	Reference
VEGF	Global deletion delays endothelial cell differentiation, impairs vascular development, and induces lethality at E8.5	(55, 56)
	Isoform-specific deletion ($VEGF_{164}$ and $VEGF_{188}$) impairs lung microvascular development and delays airspace maturation	(57)
	Postnatal inhibition decreases somatic growth and impairs alveolarization	(58)
	Decreased expression in response to hyperoxia and mechanical ventilation in numerous animal models	(60–64)
	Overexpression promotes lung angiogenesis, and inhibits hyperoxia-induced alveolar simplification and mortality in rats	(66)
FLK-1	Homozygous deletion prevents endothelial cell differentiation and blood vessel formation, and induces embryonic lethality	(53)
	Decreased expression in response to mechanical ventilation in neonatal mice	(63, 64)
	Postnatal inhibition impairs lung angiogenesis and alveolarization and induces pulmonary hypertension in neonatal rats	(8, 9)
FLT-1	Homozygous deletion causes disorganization of vascular development and induces embryonic lethality	(54)
	Decreased expression in response to mechanical ventilation in preterm baboons	(63)
NFκB	Pharmacologic inhibition in neonatal mice impairs lung angiogenesis and alveolarization and decreases *Flk-1* expression, and exaggerates the impairment in angiogenesis and alveolarization induced by systemic endotoxin	(65, 66)
HIF-1α	Global deletion results in numerous cardiac and vascular abnormalities and embryonic lethality at E10.5	(71, 72)
	Decreased expression in response to mechanical ventilation in preterm baboons and lambs	(69, 75)
	Stabilization of HIF improves alveolar growth in preterm baboons and neonatal rats exposed to combined endotoxin/hyperoxia	(78–80)
HIF-2α	Global deletion results in perinatal mortality due to respiratory failure, decreased VEGF expression, and decreased surfactant	(73)
	Decreased expression in mechanical ventilation of preterm baboons and lambs, and in neonatal rats exposed to chronic hypoxia	(69, 75)
NO/eNOS	Deletion of eNOS impairs VEGF-mediated angiogenesis and neovascularization, worsens pulmonary hypertension in adult mice exposed to chronic hypoxia, and increases susceptibility of neonatal mice to the impaired alveolarization induced by hyperoxia	(88, 90, 91, 99, 100)
	Decreased eNOS expression in mechanically ventilated preterm baboons and lambs, in fetal lambs exposed to intrauterine endotoxin	(94–96)
	Decreased NO production in pulmonary arteries from fetal lambs with intrauterine growth restriction	(97)
H_2S	Deletion of enzymes that produce H_2S impairs alveolarization, decrease lung vascular growth, and induce pathologic vascular remodeling	(104)
	Exogenous administration improves alveolarization, limits pulmonary hypertension, and decreases lung inflammation in neonatal rats and mice exposed to hyperoxia	(105, 106)
Retinoic acid	Deletion of the RA receptor-gamma impairs alveolarization and decreases lung elastin	(110)
	Promotes alveolar regeneration in adult mice with elastase-induced emphysema and limits the impaired alveolarization induced by glucocorticoids in neonatal mice	(108, 109)
LPA	Deletion of the LPA-receptor 1 limits lung inflammation and fibrosis, and improves survival in neonatal rats exposed to hyperoxia	(116)
	Pharmacologic blockade of LPA receptors -1 and -3 limits pulmonary hypertension in newborn rats exposed to hyperoxia	(116)
EC-SOD	Deletion impairs alveolarization and lung angiogenesis, and decreases FLK-1 protein expression in neonatal mice	(118)
	Alveolar epithelial overexpression preserves alveolar and vascular growth of neonatal mice exposed to hyperoxia	(119)

the predominant isoform by late alveolarization (48). Paralleling the expression pattern observed with VEGF, FLT-1 and FLK-1 are highly expressed by endothelial cells during lung development (49), and in the murine lung, the expression of both receptors increase during alveolarization and remain high in the adult lung (47, 48). In addition to the full-length, membrane-bound form of FLT-1, a soluble form, comprised of the extracellular ligand binding domain, can be produced by alternative splicing from a single gene transcript (50, 51). It is thought that this soluble form (sFLT-1) may function as a physiologic inhibitor of angiogenesis given its ability to sequester VEGF ligands and prevent them from binding to the active transmembrane receptors (52).

The absolute requirement of intact VEGF signaling for vascular development is underscored by the severe phenotypes observed in mice containing targeted disruptions of discrete components of the pathway. Homozygous deletion of *Flk-1* in mice results in early embryonic lethality, complete absence of blood vessel formation, and a failure of endothelial differentiation (53). In contrast, while homozygous deletion of *Flt-1* also results in embryonic lethality, endothelial cell differentiation is preserved, and the vasculature develops but is very disorganized (54). Targeted deletion of *Vegf* in mice delays endothelial cell differentiation and severely impairs vascular development, resulting in embryonic lethality between E8.5 and 9.5 (55). Of note, even the absence of a single allele of *Vegf* impairs vascular development and induces embryonic lethality (56). Absence of the two heparin-bound isomers, $VEGF_{164}$ and $VEGF_{188}$, impairs lung microvascular development and delays airspace maturation in mice, suggesting that these isoforms which are bound tightly in the extracellular matrix may provide a source of local VEGF specifically essential for pulmonary vascular development (57).

In addition to these indispensable roles for VEGF during embryonic development, VEGF is also an important mediator of postnatal organ growth and development. Partial inhibition of VEGF in mice during the first week of life using an inducible gene targeting strategy decreases somatic growth and impairs organ development, while complete inhibition by the administration of a soluble VEGF receptor chimeric protein exaggerates these effects on organ development and growth and specifically impairs alveolarization (58). Moreover, the spatial expression of VEGF

during late development is critical. Expression of $VEGF_{164}$ in the alveolar type II (ATII) cells using the SP-C promoter induces earlier and higher levels of VEGF in the developing lung and increases pulmonary blood vessel growth, but disrupts branching morphogenesis and inhibits alveolar type I cell differentiation (59). Taken together, these studies demonstrate the importance of tightly regulated temporal and spatial expression of VEGF for normal vascular development.

Abnormalities in VEGF signaling appear to be a key mechanism in the impaired alveolarization and angiogenesis observed in experimental models of BPD. Chronic exposure to hyperoxia in neonatal rabbits decreases VEGF gene and protein expression by alveolar epithelial cells (60). In neonatal rats, high levels of hyperoxia decrease *Vegf* gene expression (61), and sustained hyperoxia from postnatal day (P)4–14 impairs alveolarization, and suppresses *Vegf* and *Hif-2α* gene expression and VEGF receptor protein expression (61, 62). In the preterm baboon model of BPD, mechanical ventilation and oxygen reduce pulmonary capillary volume, impair alveolarization, and repress the physiologic increase in VEGF and FLT-1 observed in control animals (63). Similarly, mechanical ventilation of neonatal mice during the late saccular stage of development induces alveolar simplification and reduces lung expression of VEGF and FLK-1 (64). Inhibiting constitutive activation of nuclear factor-κB, a direct regulator of *Flk-1* during alveolarization, impairs pulmonary angiogenesis and disrupts alveolarization in neonatal mice (65), and exaggerates the impairment in angiogenesis and alveolarization induced by systemic endotoxin (66). Moreover, blocking angiogenesis in neonatal rats directly using either non-specific anti-angiogenic compounds, or a selective FLK-1 inhibitor, decreases pulmonary arterial density and impairs alveolarization, thus providing some of the first direct, experimental evidence to support the notion that angiogenesis actively promotes distal lung growth (8). In fact, even the administration of a single dose of the FLK-1 inhibitor significantly decreases pulmonary arterial density, impairs alveolarization, and induces pulmonary artery muscularization and RVH that persist into adulthood (9). Consistent with these studies, overexpression of VEGF in newborn rats is effective in increasing survival, promoting lung angiogenesis, and preventing hyperoxia-induced alveolar simplification (67).

Hypoxia-Inducible Factor

Fetal development occurs at low oxygen tension. The hypoxia-inducible factor (HIF) family of transcription factors is a key regulator of O_2 homeostasis, activating genes critical for energy metabolism, oxygen transport, and angiogenesis. The HIFs are heterodimeric transcription factors comprised of oxygen sensitive subunits (HIF-1α, HIF-2α, and HIF-3) paired with the constitutively expressed HIF-1β (previously known as ARNT) subunit. Under normal oxygen tension, the O_2 sensitive subunits are continuously degraded. However, under conditions of low oxygen tension, HIF degradation is inhibited, resulting in HIF protein stabilization and accumulation, thereby promoting the binding of HIF to hypoxia-response elements (HREs) located within the promoters of downstream target genes, including *VEGF*. During lung development, HIF-1α is expressed in the branching epithelium, and HIF-2 expressed in both the epithelium and the mesenchyme (68). In the primate lung, expression of both HIF-1α and HIF-2 is high in the third trimester of pregnancy; however, at term birth, HIF-2 expression remains high while HIF-1α is absent (69). In mouse lung, HIF-2α expression also increases immediately after birth and remains high throughout alveolarization, with production predominantly by ATII cells and colocalizing with VEGF expression (70).

The importance of this pathway in vascular development was highlighted by studies that performed targeted deletions of HIF family members in mice. Loss of *Hif-1α* results in embryonic lethality at E10.5, with null embryos demonstrating numerous cardiac and vascular malformations including vascular regression and abnormal vascular remodeling (71, 72). Interestingly, although this phenotype was similar to that seen in the VEGF null mice, *Hif-1α*$^{-/-}$ mice were found to have normal levels of *Vegf* mRNA, suggesting that the vascular malformations observed were independent of impairments in VEGF expression. In contrast, *Hif-2α*$^{-/-}$ mice die from RDS during the perinatal period in association with decreases in ATII-mediated expression of VEGF and insufficient surfactant production (73). Moreover, a similar phenotype is induced in mice by specifically deleting the HRE located within the *Vegf* promoter. Targeted deletion of ARNT, the dimerization partner for both HIF-1α and HIF-2α, as well as for other transcription factors, also results in embryonic lethality at E10.5, with affected embryos displaying defective angiogenesis of the yolk sac and branchial arteries (74).

Experimental studies in animal models of BPD suggest that HIF family members are important for late lung development in general and, in specific, that HIF plays an important role in both normal pulmonary vascular development and abnormal pulmonary vascular remodeling. HIF-1α and HIF-2α protein are decreased in the lungs of preterm baboon and lambs undergoing mechanical ventilation (69, 75). Expression of HIF-2α is also decreased in the lungs of neonatal rats exposed to chronic hypoxia, another stimulus that impairs alveolar development and decreases pulmonary vascular growth in mice (76). Enhancement of HIF signaling by either selective or non-selective inhibition of PHD-mediated HIF degradation increases angiogenesis of lung microvascular endothelial cells *in vitro*, in association with increases in PECAM-1, VEGF, and FLT-1 (77). A similar strategy to stabilize HIFs *in vivo* increases VEGF and PECAM expression in the lungs of preterm baboons (78), and improves alveolarization, oxygenation, and lung compliance (79). In a newborn rat model of BPD induced by intra-amniotic LPS followed by hyperoxia, non-selective inhibition of PHDs stabilizes HIF-1α in the whole lung, and attenuates the disrupted alveolar and vascular growth observed in this model (80). Interestingly, sildenafil, a phosphodiesterase inhibitor that has been used clinically to treat PH by increasing cGMP levels, improves alveolarization in neonatal mice exposed to hyperoxia and directly activates HIF-1α-mediated signaling in airway epithelial cells (81).

Nitric Oxide

Nitric oxide (NO) is a free radical gas that functions as a second messenger, regulating diverse physiologic processes such

as angiogenesis, vasodilation, and anticoagulation (82). NO is produced by the nitric oxide synthase (NOS) family of proteins, which contains three isoforms: neuronal NOS (*NOS1*), inducible NOS (*NOS2*), and endothelial NOS (*NOS3*). After release from the endothelium, NO can diffuse to the luminal side of the vessel to inhibit platelet aggregation and adhesion or to the abluminal side of the vessel where it regulates vascular smooth muscle contraction and proliferation (83). Many of the downstream effects of NO on vascular tone result from the ability of NO to activate soluble guanylyl cyclase, thereby increasing cGMP and decreasing intracellular calcium.

Endothelial nitric oxide synthase (eNOS), initially believed to be expressed solely by endothelial cells in a constitutive fashion, is now known to be expressed by additional cell types (84) and dynamically regulated in response to hypoxia, inflammation, and other factors (84, 85). Importantly, VEGF induces eNOS expression *via* a FLK-1-dependent mechanism (86, 87), and loss of eNOS impairs VEGF-mediated angiogenesis (88). NO is a downstream effector of VEGF-mediated angiogenesis but not fibroblast growth factor (FGF)-mediated angiogenesis (89), and *eNOS*$^{-/-}$ mice demonstrate impaired VEGF-mediated angiogenesis (88) and neovascularization during wound healing and after ischemia (90, 91). Expression of eNOS is modulated by changes in oxygen tension both *in vitro* and *in vivo*. NOS activity in pulmonary artery endothelial cells increases at higher oxygen concentrations and decreases at lower oxygen concentrations (92), an effect mediated by both transcriptional and posttranscriptional mechanisms (93).

Decreased expression of eNOS is observed in a number of animal models of BPD. Chronic ventilation of preterm lambs increases pulmonary vascular and airway resistance, and decreases eNOS protein expression in the endothelium of the small intrapulmonary arteries and the airway epithelium (94). Similarly, chronic ventilation of extremely preterm fetal baboons also decreases lung eNOS expression (95). Intra-amniotic endotoxin also decreases eNOS expression in the lungs of fetal lambs, particularly in small pulmonary arteries (96). In an ovine model, intrauterine growth restriction decreases pulmonary vascular density and alveolarization, in association with decreases in VEGF-induced NO production in large proximal pulmonary arteries (97).

In adult mice, compensatory lung growth after pneumonectomy is severely impaired by targeted deletion of eNOS or inhibition of NO production with a NOS inhibitor (98). Exposing adult *eNOS*$^{-/-}$ mice to mild hypoxia induces more severe PH than that seen in control mice (99), and exposing neonatal *eNOS*$^{-/-}$ mice to mild hypoxia impairs alveolarization and decreases pulmonary vascular density (100). In both models, these detrimental effects on pulmonary pressures and lung structure are rescued by iNO (99, 101). Further, iNO appears to have beneficial effect in other experimental models of BPD. Treatment of neonatal rats with a single dose of the FLK-1 inhibitor, SU-5416, impairs alveolarization and induces RVH, and iNO administration prevents RVH development and significantly increases radial alveolar counts (102). Prolonged iNO therapy also prevents RVH and partially rescues the severe defect in alveolarization induced by bleomycin in neonatal rats (103).

ADDITIONAL MOLECULAR MECHANISMS THAT MAY INFLUENCE ALVEOLAR AND VASCULAR GROWTH

In addition to the well-established molecular pathways described above that are central regulators of normal pulmonary vascular development and function, a number of additional molecules and pathways have been recently identified that also appear play a role in the aberrant vascular growth observed in BPD.

Hydrogen Sulfide

In addition to NO, hydrogen sulfide (H_2S) is an additional gasotransmitter that appears to have an important role in late lung development. H_2S is produced by two main enzymes: cystathionine β-synthase (Cbs) and cystathionine γ-lysase (Cth). Deletion of either *Cbs* or *Cth* decreases alveolar number by 50%, reduces the pulmonary vascular supply, and increases the number of muscularized small and medium-sized pulmonary arteries (104). In addition, H_2S appears to have important, direct effects on the angiogenic function of pulmonary endothelial cells. Silencing or pharmacologic inhibition of Cbs and Cth, respectively, impairs *in vitro* tube formation in human lung endothelial cells, and conversely, exogenous administration of H_2S enhances tube formation *in vitro* (104). Further, exogenous administration of H_2S improves alveolarization *in vivo* and limits PH in hyperoxia-exposed neonatal rats (105); and improves epithelial repair and decreases inflammation in hyperoxia-exposed neonatal mice (106).

Retinoic Acid

Retinoic acid (RA) is a biologically active derivative of vitamin A. Early studies identified a role for vitamin A and RA in enhancing limb regeneration in amphibians after amputation (107). Subsequently, RA was shown to promote alveolar regeneration in adult rats in elastase-induced emphysema (108) and to blunt the impaired alveolarization induced by dexamethasone in neonatal rats (109). Mice with genetic deletion in the RA-receptor-gamma have decreased lung elastin and impaired alveolarization (110). Pulmonary endothelial cells are a source of RA in the developing lung, where it appears to promote pulmonary angiogenesis by increasing the expression of VEGF-A and to regulate elastin synthesis by increasing FGF-18 expression (111).

Lysophosphatidic Acid

Lysophosphatidic acid is a small glycerophospholipid that exerts multiple biologic effects on cell proliferation, migration, survival, and cell–cell interactions by binding to G-protein coupled receptors on the cell membrane (112). LPA appears to have an important role in many lung diseases, functioning to regulate airway inflammation, remodeling, and fibrosis (113–115). In the vasculature, LPA can function as either a vasodilator or a vasopressor depending on context. For example, in the thoracic aorta, LPA causes NOS-dependent vasodilation by acting through the LPA receptor-1 (LPAR1). Mice containing mutations in the LPAR1 demonstrate decreased lung inflammation and fibrosis

and improved survival in an experimental model of BPD, and pharmacologic blockade of the LPAR-1 and -3 protects against pathologic vascular remodeling, limiting muscularization and RVH in newborn rats exposed to chronic hyperoxia (116). Although there were some phenotypic differences between the mice with genetic deletions of LPAR-1 and pharmacologic blockade that require future study, these studies suggest that the LPA pathway may prove to be a promising new target for BPD.

Extracellular Superoxide Dismutase

Extracellular superoxide dismutase (EC-SOD) is a potent antioxidant that catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen (117). EC-SOD is highly expressed in the lung and vasculature, and EC-SOD expression and activity is suppressed in experimental models of BPD (118). Alveolar epithelial overexpression of EC-SOD preserves alveolar surface and volume density, decreases inflammation in newborn mice exposed to hyperoxia (119), and attenuates pathologic vascular remodeling and PH in adult mice exposed to chronic hypoxia (120). Conversely, deletion of EC-SOD impairs alveolarization in neonatal mice and decreases pulmonary vascular density and Flk-1 protein expression (118). Taken together, these studies highlight the importance of tight control of the oxidative balance in the lung in promoting physiologic alveolar and vascular growth, and preventing pathologic airway and vascular remodeling.

Stem and Progenitor Cells

A number of resident stem and progenitor cell populations have been identified in the lung, deriving from epithelial, mesenchymal, and endothelial origins. Each population is unique in its defining characteristics and putative functions, which are comprehensively discussed in a number of excellent, recent reviews (121–123). Accumulating evidence from clinical and experimental studies have suggested that alterations in circulating and/or resident lung stem and progenitor cells may contribute to the pathogenesis of BPD, sparking great interest in the investigation of cell-based therapeutic strategies as a potential treatment for BPD. Hyperoxia decreases lung and circulating endothelial progenitor cells in neonatal mice (124), and diminishes the number of lung side population (SP) progenitor cells, a population believed to have both epithelial and mesenchymal potential (125). Further, studies in experimental models suggest that mesenchymal stem cell therapy may have beneficial effects on preserving alveolar and vascular growth during injury. Intratracheal administration of mesenchymal stem cells attenuates induced lung cell apoptosis and inflammation, and improves alveolarization in neonatal rats exposed to hyperoxia (126). Intravenous administration of bone marrow-derived mesenchymal stem cells (BMSCs) in neonatal mice prevents PH and blunts the impaired alveolarization induced by hyperoxia despite a low level of engraftment. Importantly, in that study, the administration of conditioned media of these stem cells had an even greater beneficial effect, preserving normal alveolarization and preventing pathologic vascular remodeling (127). A similar improvement in alveolar and vascular growth is observed in hyperoxia-exposed neonatal rats after intratracheal administration of BMSCs (128), and this beneficial effect is evident even if the MSCs are administered after the initiation of lung injury (129). Moreover, MSC treatment results in durable improvements in lung structure, with sustained improvement in lung structure and exercise tolerance in adult mice at 6 months of age, and an absence of any evidence of long-term detrimental side effects. These exciting data prompted clinical studies to assess whether alterations in lung progenitor cells play a role in BPD, discussed in the following section.

ALTERATIONS IN ANGIOGENIC PATHWAYS IN PATIENTS WITH BRONCHOPULMONARY DYSPLASIA

These data, obtained from experimental models demonstrating disruption of key pathways known to promote physiologic pulmonary angiogenesis, appear to have some fidelity with the human disease. The impaired pulmonary vascular development observed in infants dying of severe BPD is associated with decreased expression of VEGF and FLT-1 (6). In response to short-term ventilation, the expression of classic angiogenic growth factors, such as VEGF and angiopoietin-1, decreases in the lungs of preterm infants, while expression of endoglin increases, suggesting that endoglin may be one important regulator of the vascular remodeling which occurs in BPD (130). In a similar, but separate, study by the same group, short-term ventilation decreases the gene expression of proangiogenic factors such as *FLK-1*, TEK tyrosine kinase, endothelial (*TIE-2)*, and angiogenin, yet increases the expression of anti-angiogenic mediators such as thrombospondin-1 (131). Taken together, these two studies suggest that even short-term mechanical ventilation causes widespread alterations in a variety of angiogenic signaling pathways in the developing lung.

In contrast to these studies demonstrating changes in the gene and protein expression of angiogenic mediators from whole lung tissue of patients dying with BPD, studies evaluating levels of VEGF in the tracheal fluid have not shown clear differences between preterm infants who develop and those who do not develop BPD. Lassus et al. found that the levels of VEGF in tracheal fluid obtained during the first 10 days of life are not significantly different in preterm infants who developed BPD versus those who do not develop BPD (132). In keeping with these results, two additional studies demonstrated that tracheal fluid VEGF levels obtained during the first month of life also did not correlate with the development of BPD (133). However, it is not clear whether the absence of positive findings in these studies represent differences between the pathogenesis of experimental BPD and the human disease, a lack of statistical power, or the inability of tracheal aspirates to reflect the true microenvironment present in the developing lung.

Similarly, despite strong experimental evidence demonstrating the importance of both the HIF and NO signaling pathways in physiologic pulmonary angiogenesis, data assessing the integrity of the HIF of NO signaling in patients with BPD remain scarce. In the developing human lung, both *HIF-2α* and *VEGFA* gene expression demonstrate a positive correlation as lung development progresses; however, little is known regarding how HIF

activity or expression is altered in preterm infants with BPD. Similarly, there is an absence of data directly demonstrating decreased NOS expression or NO production in infants with BPD. However, the levels of the endogenous NOS inhibitor, asymmetric dimethylarginine (ADMA), are increased in patients with BPD and PH, suggesting that heightened levels of ADMA may contribute to the increased PVR observed in patients with BPD and PH by limiting NO production (134). Yet, despite extensive experimental evidence demonstrating disruptions in NO signaling and the therapeutic benefit of iNO therapy in animal models, a number of recent, prospective, and randomized trials have failed to demonstrate beneficial effects of iNO therapy in the prevention of BPD in preterm infants (135–137).

Given the accumulating evidence from experimental models that demonstrated the beneficial role of stem and progenitor cells in promoting alveolar and vascular growth during injury, clinical studies aimed to determine whether disruption of angiogenic progenitors might contribute to the pathophysiology of BPD. Late outgrowth endothelial colony-forming cells (ECFCs), a sub-type of EPCs that are highly proliferative, self-renewing, and capable of forming blood vessels *de novo in vivo* (138). ECFCs obtained from preterm infants are more proliferative than those obtained from term infants, yet more highly susceptible to the growth inhibiting effects of hyperoxia (139). In a small, early prospective study, ECFCs were found to be low in extremely premature infants and to increase with increasing gestation. Further, extremely preterm infants with lower numbers of ECFC were found to be at increased risk of developing BPD (140). In keeping with these results, a subsequent study confirmed that cord blood ECFCs are significantly lower in preterm infants who go onto develop moderate or severe BPD (141). Taken together, these studies lend further support to the notion that antenatal events may influence later respiratory outcomes, and suggest that ECFC may represent a biomarker for the identification of patients at greatest risk for the development of BPD. In addition to these endothelial progenitors, another small clinical study demonstrated the presence of fibroblast-like cells with colony-forming potential and cell surface marked similar to MSCs in the tracheal aspirates of premature infants with RDS. After adjusting for numerous potential confounders, including gestational age, duration of mechanical ventilation, and others, the presence of these tracheal MSC predicted the development of BPD (142). Although clinical evidence regarding the role of MSC in patients with BPD is limited, the strong experimental evidence demonstrating the benefit of MSC therapy on alveolar and vascular growth in animal models has already lead the way for phase 1 clinical trails for testing this therapy in preterm infants at high risk for BPD (143).

CONCLUSION

Over the past three decades, significant advances in the supportive care of extremely premature infants, including surfactant replacement therapy, have significantly decreased mortality from BPD, yet, the morbidity associated with BPD remains high. Numerous abnormalities of the pulmonary circulation are observed in patients with BPD, influencing long-term prognosis, including dysmorphic pulmonary capillary development, maladaptive pulmonary vascular remodeling, heighted pulmonary vascular tone, and the development of abnormal collateral circulation. Extensive experimental and clinical data derived form studies over the last decade have advanced our understanding of the pathobiology contributing to BPD, including the recognition that pulmonary angiogenesis is essential for alveolarization, and that disrupted pulmonary angiogenesis likely contributes to BPD. Given the limited availability of human lung tissue from patients with BPD, much of our understanding of the molecular mechanisms involved have been derived from experimental animal models (144), and definitive clinical evidence demonstrating that these same mechanisms are causative in the human disease are lacking. Nonetheless, these studies suggest that replacement of angiogenic factors and/or stem cell-based therapies could prove to be beneficial for the treatment of BPD. Moving forward, the development of innovative non-invasive diagnostic technologies that may permit an accurate assessment of the molecular pathways that are dysregulated in patients at risk for BPD will be required in order to foster the development of targeted biologic therapies that can effectively stimulate lung growth and regeneration.

AUTHOR CONTRIBUTIONS

Dr. CA composed the manuscript and designed the figure and table.

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REVIEW
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The Future of Bronchopulmonary Dysplasia: Emerging Pathophysiological Concepts and Potential New Avenues of Treatment

Jennifer J. P. Collins[1], Dick Tibboel[1], Ismé M. de Kleer[2], Irwin K. M. Reiss[3] and Robbert J. Rottier[1]*

[1] Department of Pediatric Surgery, Sophia Children's Hospital, Erasmus University Medical Centre, Rotterdam, Netherlands, [2] Division of Pediatric Pulmonology, Department of Pediatrics, Sophia Children's Hospital, Erasmus University Medical Centre, Rotterdam, Netherlands, [3] Division of Neonatology, Department of Pediatrics, Sophia Children's Hospital, Erasmus University Medical Centre, Rotterdam, Netherlands

Yearly more than 15 million babies are born premature (<37 weeks gestational age), accounting for more than 1 in 10 births worldwide. Lung injury caused by maternal chorioamnionitis or preeclampsia, postnatal ventilation, hyperoxia, or inflammation can lead to the development of bronchopulmonary dysplasia (BPD), one of the most common adverse outcomes in these preterm neonates. BPD patients have an arrest in alveolar and microvascular development and more frequently develop asthma and early-onset emphysema as they age. Understanding how the alveoli develop, and repair, and regenerate after injury is critical for the development of therapies, as unfortunately there is still no cure for BPD. In this review, we aim to provide an overview of emerging new concepts in the understanding of perinatal lung development and injury from a molecular and cellular point of view and how this is paving the way for new therapeutic options to prevent or treat BPD, as well as a reflection on current treatment procedures.

Keywords: bronchopulmonary dysplasia, chronic lung disease of prematurity, respiratory distress syndrome, preterm birth, lung development, chronic lung disease

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****Correspondence:***
Jennifer J. P. Collins
j.dewolf-collins@erasmusmc.nl

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INTRODUCTION

Yearly over 15 million babies are born premature (<37 weeks gestational age), accounting for more than 1 in 10 births worldwide, of which approximately 2.4 million babies are born before 32 weeks of postmenstrual age (PMA) (1). Bronchopulmonary dysplasia (BPD) is the most common adverse outcome in very preterm neonates with an incidence of 5–68%, depending on the cohort and definition used, which increases significantly with declining gestational age (2, 3). BPD develops as a result of lung injury caused by maternal pre-eclampsia, chorioamnionitis, postnatal ventilation, hyperoxia, and/or inflammation, leading to an arrest in alveolar and microvascular development and pulmonary hypertension, although the relative contribution of the different pathogenic factors for the individual patient is hard to identify (4). Originally, BPD ("old" BPD) was defined based on lung injury resulting from mechanical ventilation and oxygen supplementation, and was seen mostly in premature infants born at 26–30 weeks PMA (5–7). The introduction of major interventions such as maternal corticosteroids (8, 9) and surfactant replacement therapy (10–12) resulted in a changed disease phenotype that was seen in preterm infants that could survive at younger gestational

ages (24 to 26 weeks PMA). As a result, "new" BPD, defined as the requirement of supplemental oxygen at 36 weeks PMA or treatment with supplemental oxygen for more than 28 days (4), was characterized based on impaired alveolar and capillary development of the immature lungs (13). It is now becoming clear that BPD survivors continue to have respiratory morbidity after they leave the neonatal intensive care unit (NICU) [see comprehensive review by Islam et al. (14)], underlining that BPD really is a disease of disrupted lung development. Understanding how the alveoli and underlying capillary network develop and how these mechanisms are disrupted in BPD is critical for developing efficient therapies, which currently are lacking. Moreover, the nature of lung injury and consequently BPD is perpetually changing as treatment strategies evolve in an attempt to prevent injury to the premature lungs. Combined with increasing insight into the pathophysiology of BPD, this has started a discussion on yet a newer definition of what BPD is, basing it more on biomarkers, pulmonary hypertension and the underlying vascular basis of BPD (15–17). In this review, we provide an overview of emerging new pathophysiological concepts in the understanding of perinatal lung development and injury from a molecular and cellular point of view and how this is paving the way for new therapeutic options to prevent or treat BPD, as well as a reflection on how this compares with current treatment procedures.

Overview of Lung Development

To understand BPD pathophysiology, it is important to understand how the lung normally develops. Despite the large body of knowledge concerning the morphogenesis of the lung (18, 19), research on the intercellular communications that regulate growth, migration, and differentiation during lung development is still unfolding. Among the best characterized growth factors and their signaling components in early lung development are fibroblast growth factor (FGF), transforming growth factor β (TGFβ), bone morphogenetic protein (BMP), sonic hedgehog (SHH), wingless-type MMTV integration site family (WNT), vascular endothelial growth factor (VEGF), and retinoic acid signaling pathways [reviewed by Hogan and Morrissey (20) and Kool et al. (21)]. Far less is known about the molecular and cellular processes that direct saccular and alveolar development, the very stages that are clinically relevant after preterm birth and BPD pathogenesis. VEGF, which is expressed by alveolar epithelial type II cells in response to hypoxia-induced factor (HIF), is crucial in directing pulmonary microvascular development and alveolar development (22). Moreover, VEGF plays an important role in BPD pathogenesis as BPD patients express little or no VEGF in their lung epithelium, and lack expression of VEGF receptors in pulmonary microvascular endothelium (23). Multiple studies have demonstrated that platelet derived growth factor (PDGF) and FGF signaling is crucial for myofibroblast differentiation and subsequent onset of secondary septation (24–29). WNT, BMP, and TGFβ signaling components have also been implicated to play a role in fibroblast differentiation during alveolarization (30–32). Additionally, correct deposition of extracellular matrix (ECM) proteins by myofibroblasts, like elastin and collagen, plays a crucial role during secondary septation (33, 34). These and other ECM components may exert their role in lung development by functioning as a scaffold for the growth factors to coordinate the growth interactions of cells (35).

BPD IN 2017

Current Understanding of Perinatal Risk Factors

Because BPD is still very much a functional diagnosis, which is made when preterm infants have already been exposed to a wide variety of perinatal stressors [**Figure 1**; (36)], it is hard to pinpoint exactly which exposure is more detrimental for lung development. Most of these insights have been obtained through decades of work on animal models [reviewed by Jobe (37)] and correlations found through epidemiological research. Already before preterm birth, intrauterine conditions can have a profound impact on lung development and susceptibility to BPD. Risk factors established by statistical correlation are first and foremost maternal risk factors associated with preterm birth, such as

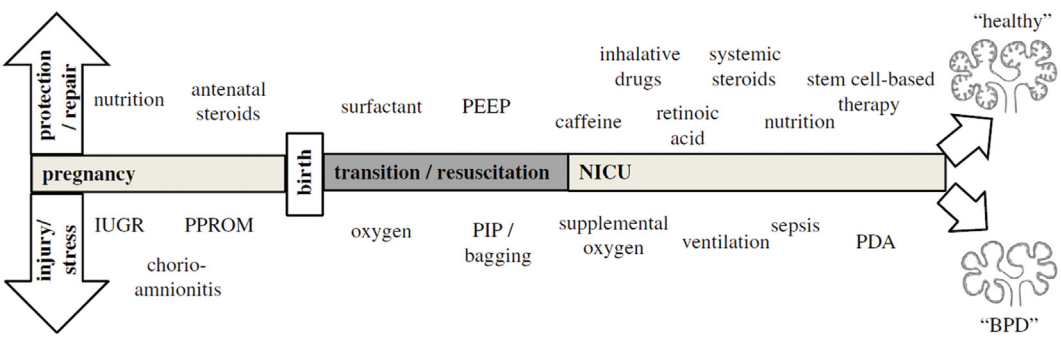

FIGURE 1 | The pathogenesis of bronchopulmonary dysplasia (BPD) is highly multifactorial in nature, with a wide variety of pre- and postnatal exposures influencing lung development. Depending on the timing and combinations of exposures, BPD likely exists of multiple different pathophysiologies that manifest themselves in a similar way clinically. The top arrow represents exposures that may to a certain extent protect from BPD pathogenesis and promote repair, while the bottom arrow indicates exposures that injure the preterm lung and contribute to BPD pathogenesis. Figure reprinted from Hütten et al., originally published by Springer (36).

smoking and socioeconomic background (38). Intrauterine growth restriction increases the risk of BPD threefold in infants born before 29 weeks (2, 39), while chorioamnionitis and pre-eclampsia trigger the release of cytokines and growth factors that directly inhibit alveolar and microvascular development of the fetal lungs (2, 36, 40). Placental abnormalities, such as gestational hypertension, pre-eclampsia, and eclampsia, are emerging as an important antenatal risk factor for BPD. A French prospective cohort study found that placenta-mediated pregnancy complications with fetal consequences are associated with moderate to severe BPD in very preterm infants (41). The maternal administration of corticosteroids prior to preterm birth leads to thinning of the primary septa, which narrows the air blood barrier, stimulates the production of surfactant, which stabilizes the alveolar sacs and prevents collapse after exhalation, and stimulates the clearance of fetal lung fluid (42). Although this accelerated development improves neonatal outcome and survival of the infant, antenatal corticosteroids have the unwanted side effect of inhibiting secondary septation and impairing microvasculature development (28, 43–45).

Postnatally, inflammation is also considered to be an important risk factor for the development of BPD [reviewed in Ref (46)], either as a result of lung injury caused by invasive mechanical ventilation and supplemental oxygen or in the form of sepsis. Due to their lung immaturity and apnea of prematurity, preterm infants are also frequently exposed to hypoxia, which just like hyperoxia leads to impaired alveolar and microvascular development (47). Recently, the presence of oxygen-sensitive intrapulmonary bronchopulmonary anastomoses (IBA) was discovered in preterm infants with BPD and other infants with chronic lung diseases, which may stay patent in the setting of persistent hypoxia (16, 48–52). Thus, IBA may in itself lead to persistent hypoxemia and contribute to the pulmonary hypertension that is often seen in conjunction with BPD, and could therefore be a significant risk factor for BPD (16). Considering that not all infants that are born very or extremely preterm go on to develop BPD, multiple pre- and/or postnatal hits are probably needed for lung development to be significantly affected, especially since the incidence of BPD has not decreased despite advances in neonatal care (2).

Current Treatment Procedures

In this complex multifactorial setting, current therapies are aimed to not only support the survival of the preterm infant, but also to limit or prevent further damage as much as possible [see review by Jain and Bancalari (53)]. In this regard, the most direct approach is to prevent the need for aggressive, prolonged invasive ventilation. The first treatment of choice to prevent respiratory distress syndrome (RDS) is still antenatal maternal corticosteroid administration, followed by prophylactic surfactant therapy through endotracheal bolus administration after birth. The maternal administration of a single or repeated intramuscular injection of betamethasone or dexamethasone within a time window of 24 h to 7 days prior to preterm birth can significantly increase survival of the preterm infant and decrease the incidence and severity of RDS (9, 54). However, there is no consensus yet on how the use of antenatal steroids can be optimized by improving the timing of administration and dosing (42). Similarly, there is discussion as to whether surfactant therapy should be prophylactic or only selectively administered upon diagnosed RDS, as a result of the increased use of non-invasive ventilation methods such as nasal continuous positive airway pressure (CPAP) (53, 55). Without the application of routine CPAP, prophylactic surfactant treatment reduces neonatal mortality. However, the routine application of CPAP reduces the risk of BPD and neonatal death, and in these infants selective administration of surfactant is more beneficial (55). The INSURE method (intubate-surfactant-extubate to CPAP) is therefore now the recommended technique to avoid lung injury (56).

An alternative method of surfactant administration that builds on this is less invasive surfactant administration (LISA), which circumvents the need of endotracheal intubation and mechanical ventilation all together while improving pulmonary outcome in extreme premature infants (57–59). A more high-tech approach that is now being tested in the NICU is surfactant administration through aerosolization, nebulization, or atomization (60–67). It has proven technically challenging to achieve sufficient delivery of surfactant in the distal lung compared to bolus administration of surfactant, although the recent development of vibrating membrane nebulizers seems promising (67). Switching from animal-derived surfactants to new generation synthetic surfactants, which are more resistant to inactivation and even anti-inflammatory in cell culture and animal studies, may be another step forward (11, 68–75). Several clinical trials are testing two promising synthetic surfactants to combat RDS in the NICU. A multicenter phase 2 study is comparing the safety and efficacy of CHF5633, a synthetic surfactant with surfactant protein (SP)-B and SP-C analogs, with poractant alfa in preterm infants with RDS (ClinicalTrials.gov identifier NCT02452476). In addition, two multicenter phase 2 studies are assessing the safety and efficacy of aerosolized lucinactant (also known as KL4 surfactant, Aerosurf, and Surfaxin) in preterm neonates 26 to 32 weeks PMA receiving nasal CPAP (ClinicalTrials.gov identifiers NCT02636868 and NCT02528318). Optimizing ventilation strategies and surfactant therapy are therefore seen as the most easily achievable targets in the prevention of BPD.

Besides ventilation strategies, surfactant therapy and corticosteroids, there are a few therapies that have a profound effect in the prevention of BPD. Prophylactic caffeine therapy is recommended to counter apnea of prematurity and is now common practice after it was shown to be effective in reducing BPD and subsequent neurodisability (56, 76–78). The protective effect of caffeine therapy appears greater when given earlier rather than later, although there is still discussion among experts as early therapy is also associated with slightly greater mortality in some studies (79–81). This effect has been attributed to infants receiving earlier extubation and subsequently shorter mechanical ventilation times, alleviating the injury burden on the developing premature lung (76, 79). Multiple recent animal studies have attempted to elucidate whether caffeine itself can promote or protect alveolar development directly, with mixed results. Using the hyperoxia model of experimental BPD, caffeine could protect against alveolar simplification and inflammation in rats (82, 83) and rabbits (84), but not in mice (85, 86). Potential mechanisms include its

abilities to amplify glucocorticoid-mediated SP-B expression in alveolar type 2 cells (87, 88), to modulate connective tissue growth factor (CTGF) expression (89) and TGFβ pathway members (85), and to attenuate endoplasmic reticulum (ER) stress (82). Conflictingly, both up- and downregulation of alveolar apoptosis has been reported (82, 86). Caffeine is however primarily known as a methylxanthine, which is a non-selective phosphodiesterase (PDE) inhibitor (78). PDE inhibitors have potent immunomodulatory and vascular effects and are therefore still interesting targets for neonatal intensive care medicine. Animal studies using the neonatal rodent hyperoxia model of experimental BPD have shown promise for non-selective PDE inhibitor pentoxyfilline (90), PDE4 inhibitors rolipram, piclamilast, and cilomilast (91–93), and PDE5 inhibitor sildenafil (94), which were able to ameliorate pulmonary inflammation and hypertension and improve lung alveolarization. Inhaled nitric oxide (iNO) therapy, which has a complementary mode of action to PDE inhibitors by boosting cyclic guanosine monophosphate (cGMP) (95), has long been the subject of clinical trials after promising results in animal models of BPD. Although iNO decreases inflammatory mediators in tracheal aspirates of treated preterm infants (96), systematic reviews show no protective effect in the development of BPD (97). Interestingly, iNO therapy was effective in reducing BPD incidence when combined with vitamin A therapy (98). Supplementation with vitamin A improved alveolarization in neonatal rats and lambs (99, 100), while in clinical studies, supplementation with vitamin A in preterm infants significantly reduced the risk of BPD (101–103). Unfortunately, these studies have not lead to the adoption of vitamin A supplementation in clinical practice, as the treatment benefits were deemed too small and the intramuscular route of administration too cumbersome in tiny preterm infants (104, 105). Other administration routes must be investigated for these promising therapies to become commonplace in the clinic.

For all currently used therapies, there is still ground to be gained through clinical trials and evidence-based medicine to ascertain optimal dosing, timing, and administration methods for maximum efficiency. It is essential that risk stratification takes place within the trial design to identify the real potential advantage of the different interventions. Despite all efforts at reducing lung injury through current treatment procedures, the incidence of BPD has remained stable over the past two decades (2). This is in part explained by the increased survival of extremely preterm infants born between 22 and 26 weeks PMA but probably also reflects the highly multifactorial nature of BPD. Prematurity is often not the first complication leading to BPD pathogenesis, as infants have already been exposed to a disadvantageous intrauterine environment, either through severe intrauterine growth restriction resulting from severe pre-eclampsia or chorioamnionitis. This is then followed by various exposures and comorbidities in the NICU, which in a substantial portion of these extreme premature infants leads to BPD with a similar phenotype, even though the underlying pathogenesis might have been quite different. It should not be forgotten that an astonishing portion of these infants does not go on to develop BPD, despite experiencing similar exposures. A better understanding of the pathophysiology leading to BPD is therefore crucial to create a better tailored treatment regimen for premature infants.

CURRENT UNDERSTANDING OF BPD PATHOPHYSIOLOGY, NEW PATHOPHYSIOLOGICAL CONCEPTS, AND POTENTIAL THERAPIES

Infants at greatest risk of developing BPD are born when their developing lungs are still transitioning from the canalicular to saccular phase. Given the complexity of lung development and the wide variety of perinatal insults leading to BPD, there is likely no single pathophysiology of BPD. Because of a paucity of histopathological data from preterm infants and BPD patients, our current understanding of BPD pathophysiology has mostly been generated from various small and large animal models looking at the effect of perinatal inflammation, oxygen toxicity, and mechanical ventilation on lung development [reviewed by Jobe (37)]. Although these simplified animal models of BPD only approximate the actual disease in humans, they have helped us immensely to better understand the pathophysiology of BPD. A number of recent reviews have generated a detailed overview of the various pathophysiological mechanisms implicated in BPD that have been uncovered through these models [see review by Niedermaier and Hilgendorff (106) and Hilgendorff and O'Reilly (107)], focusing on the role of perinatal infection and inflammation (46, 108, 109), pulmonary vascular development (17), the mesenchyme (110), the extracellular matrix (111), and oxygen (112) [**Figure 2** (107)]. For the remainder of this review, we will highlight new pathophysiological concepts that are promising avenues for potential future therapies for BPD. Because of the inherent intertwinement of the pathophysiological mechanisms and potential therapies, we have chosen to present these side by side for each pathophysiological concept.

Stem Cells in Development and for Therapy of BPD

In the past decade, the field of stem cell biology has advanced significantly, especially with respect to tissue resident stem cells in development and repair. A wide variety of lung epithelial stem/progenitor cells has been described but also multipotent mesenchymal stromal cells (MSCs) and endothelial colony forming cells (ECFCs) [reviewed in Ref (113)]. In the developing lung, where an extensive microvasculature is crucial for lung function, resident lung MSCs (L-MSCs) are a heterogeneous progenitor population, which orchestrate the formation of the alveolar microvasculature, repair/regeneration, and tissue maintenance [reviewed in Ref (114, 115)]. Already at the beginning of lung budding, a multipotent cardiopulmonary mesoderm progenitor has been described, based on expression of Wnt2, Gli1 and Isl1, giving rise to pulmonary vascular and airway smooth muscle, proximal vascular endothelium and pericyte-like cells (116). During pseudoglandular lung development early $Tbx4^+$ multipotent MSCs give rise to a wide variety of distinct mesenchymal cell populations including airway and vascular smooth muscle and early fibroblast-like cells (117), reminiscent of quintipotential

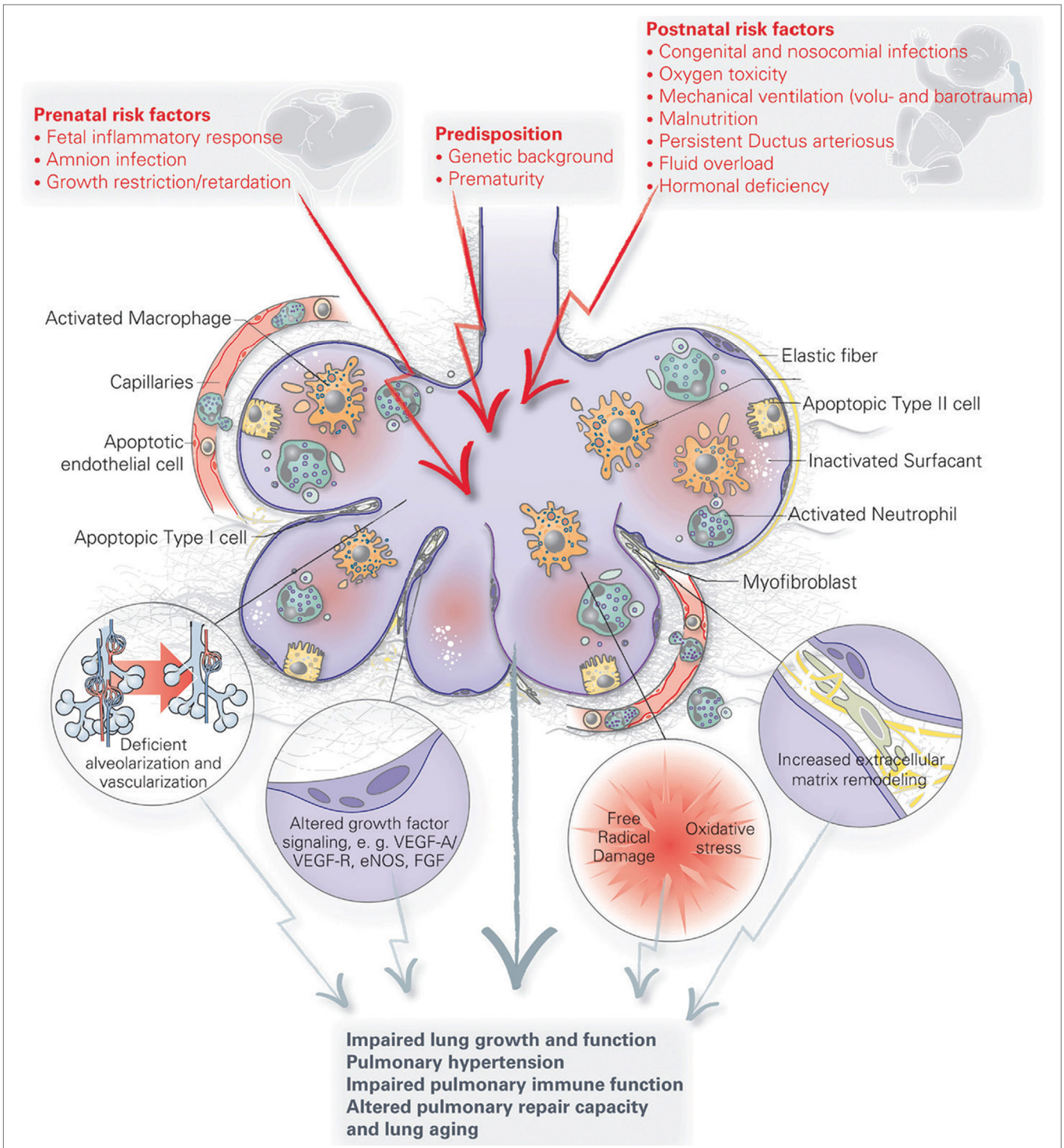

FIGURE 2 | A schematic overview of the pathophysiology of bronchopulmonary dysplasia (BPD). Pre- and postnatal risk factors lead to lung injury, resulting in apoptosis of distal lung cells, inflammation, extracellular matrix remodeling and altered growth factor signaling. These have long term effects on lung growth and function, including vascular and immune function, resulting in an increased disposition for chronic lung disorders. Figure reprinted from Hilgendorff and O'Reilly, originally published by Frontiers in Medicine (107).

MSCs in bone marrow (118). During saccular and alveolar lung development, *Pdgfrα*$^+$, *Shh*$^+$, and *Fgf10*$^+$ L-MSCs give rise to myofibroblasts and lipofibroblasts, which are crucial for alveolar development (119–122). Importantly, *Pdgfrα* + L-MSCs are supportive of lung epithelial progenitor cells, which are unable to form colonies in their absence or in the presence of more differentiated

myofibroblasts (123, 124). There is mounting evidence from both human patients and animal models that L-MSCs are perturbed in BPD, potentially actively contributing to BPD pathogenesis. The presence of L-MSCs in tracheal aspirates from ventilated preterm infants could predict the subsequent development of BPD (125). *In vitro*, these L-MSCs showed signs of dysfunction through reduced PDGFRα expression, a propensity toward myofibroblast differentiation and impaired migration capacity (126, 127). This is supported by a recent study in neonatal mice, where suppression of *Fgf10* expression left alveolar epithelial type 2 cells (AEC2) unable to regenerate after hyperoxia damage, leading to increased AEC1 differentiation (128). Combined with prior observations in parabronchial smooth muscle cells upon naphthalene injury (129), the secretion of FGF10 to stimulate epithelial repair may be one of the ways through which L-MSCs exert their regenerative capacities in the distal lung following injury (130).

Similarly, lung resident ECFCs, which are important for the development of the pulmonary microvasculature, were shown to be dysfunctional in a neonatal rat model of BPD (131). Moreover, the cord blood of preterm infants who go on to develop BPD contains lower numbers of circulating ECFCs, which are more vulnerable to hyperoxia-induced oxidative stress and dysfunction (132). Understanding how these resident progenitor populations are affected in BPD, but also how they normally mediate development, repair, and regeneration in the lung, will provide an insight into how we may mobilize these cells to actively engage in repair and normalize lung development.

Potential Therapies

Tapping into and stimulating the regenerative properties of L-MSCs and ECFCs through cell-based therapy may be a central way to ameliorate the lung injury leading to BPD pathogenesis. To this end, important lessons will come from exogenous stem cell therapy. In a neonatal rat hyperoxia model of BPD, intratracheal installation of either bone marrow or umbilical cord derived MSCs, or their conditioned media, could nearly completely repair experimental BPD, both on a histological and on a functional level (133, 134). The mode of action appears to be largely paracrine, as injection with MSC conditioned medium could promote alternatively activated (M2) macrophages (135). Exosomes, which are extracellular vesicles containing a cocktail of proteins, RNAs and even mitochondria, are secreted by a wide variety of cells including MSCs and likely play an active role in the paracrine therapeutic effects of MSCs (136). Their potential as a carrier of therapeutic paracrine factors makes them appealing and promising targets for cell-free MSC based therapy. However, several technical challenges must be overcome to ensure their safety, such as a robust reproducible isolation technique and their ability to facilitate infectious or damaging particles (137). The next decade will likely see large advances in the development of exogenous stem cell therapy for BPD and a vast array of other diseases, either by injecting stem cells themselves, their conditioned medium or through exosomes [see recent reviews by Möbius and Thébaud (138), O'Reilly and Thébaud (139), and Mitsialis and Kourembanas (136)].

Pulmonary Macrophages Contribute to Alveolar Development and Repair

Arguably the most important immune cells to participate in wound repair are alternatively activated macrophages. Besides peripheral blood derived macrophages, the pulmonary microenvironment contains three distinct resident pulmonary macrophage populations: alveolar macrophages, interstitial macrophages and primitive macrophages (140). Alveolar macrophages are the best-studied subset and are most abundantly present in the lung. They reside in the alveolar spaces where they phagocytose foreign particles and have a crucial role in the surfactant metabolism that facilitates alveolar function and gas exchange. Interstitial macrophages (IMF) reside on the other side of the epithelial barrier, among mesenchymal cells and capillaries. They have a distinct phenotype and behavior from alveolar macrophages and are geared more toward tissue repair and maintenance, antigen presentation and influencing dendritic cell functions to prevent allergy (140, 141). The third population, primitive macrophages, has only very recently been identified as a distinct subtype. These macrophages are the first to colonize the fetal lungs, and persist in adult lungs in the parenchyma of the peripheral alveoli. Because of their location in peripheral and perivascular spaces, which have been described as hotspots for alveolar regeneration, they are speculated to promote or be attracted to stem cell activity (140). The influx of these macrophages, which display an alternatively activated or M2 phenotype, and localization at the branching sites of the developing lung, suggest they potentially contribute to alveolar lung development (142). Conversely, if fetal lung macrophages are activated by an inflammatory stimulus, they actively inhibit expression of genes critical for lung development, leading to disrupted airway development and perinatal death in mice (143).

Potential Therapies

These insights provide new support for anti-inflammatory treatments. Furthermore, exogenous MSC therapy may be beneficial in regulating pulmonary macrophage activity. As cells with potent immunomodulatory capacities, MSCs can regulate macrophage function and polarization (144). Steady-state MSCs drive macrophages toward a wound healing or M2 phenotype through the production of IL-6 and inhibit differentiation toward dendritic cells (145, 146). However, in a proinflammatory environment MSCs stimulate macrophages toward a pro-inflammatory M1 phenotype (147). Using cell-based therapy to activate resident L-MSCs may therefore also be effective in promoting an M2 phenotype in pulmonary macrophages.

The Lung Microbiome: An Important Emerging Field of Interest

Although there has been a surge in interest in the microbiome thanks to the Human Microbiome Project, the lung was not included in this research project. Research interest in the lung microbiome is now however on the rise, uncovering that not only the upper but also the lower airways are colonized, with numbers of 10–100 bacterial cells per 1,000 human cells being reported (148). The six most commonly detected bacterial phyla

are found throughout the body, but composition varies per organ. In the lung, composition varies between different areas, making consistent sampling of the same area extremely important when comparing between groups. The lungs of newborn infants are already colonized at birth with a variety of bacterial phyla, most predominately *Acinetobacter* (149). The composition of the lung microbiome changes and stabilizes in the first month of life, but is decidedly different in lungs of children and adult patients with lung disease (148, 149). Interestingly, amniotic fluid and the placenta harbor their own microbiota, suggesting that fetal tissues already get colonized *in utero*, potentially having an effect on early immune cell maturation (148).

Inflammation frequently occurs in preterm infants, both antenatal (chorioamnionitis) and postnatal (sepsis), and can strongly perturb lung development (150). In the neonatal period, the immune system is still immature, and evidence is mounting that host-microbial interactions are necessary for development and homeostatic control of the immune system (151). Recently, a strong correlation was found between decreased diversity of the lung microbiome at the time of birth in preterm infants and the development of BPD (149, 152). Other studies correlated prolonged antibiotics use during the first week of life and BPD (153, 154). The protective effect of bacterial exposure in early life on asthma and allergy development, the "hygiene hypothesis," is extensively studied, and a greater microbial diversity of commensal bacteria seems to underlie this protective effect (148). Beyond microbial diversity and exposure, the role of the lung microbiome in the regulation and maturation of the immature immune system and the developing neonatal lung is less clear. One route of how the lung microbiome might train the immature immune system is by inducing expression of programmed death ligand 1 (PD-L1) in pulmonary dendritic cells. Lack of microbial colonization, or blocking pulmonary PD-L1 during the first 2 weeks of life in mice, induced a disproportionate inflammatory response to allergens later in life (155).

An imbalanced microbiome, called dysbiosis, may further impact the inflammatory and tissue repair response to oxygen exposure, as beneficial bacteria are lost or overrun by other bacteria. An important emerging mechanism through which the microbiome can influence cell function is through the production of microbial metabolites, such as short chain fatty acids or tryptophane catabolites (156, 157). Tryptophane catabolites are produced via the enzyme indoleamine 2,3-dioxygenase 1 (IDO1) and function as agonists for the aryl hydrocarbon receptor (AhR). AhR activation leads to an immune suppressive response through the production of interleukin (IL) 22 and promotes development of regulatory T-cells (158). One genus of bacteria capable of metabolizing tryptophane into AhR agonists are Lactobacilli. The beneficial effects of tryptophane metabolites and Lactobacilli have been shown to inhibit inflammation and promote health in the gut, central nervous system and the lung (156, 157). Treatment of COPD patients with emphysema with the anti-inflammatory macrolide antibiotic azithromycin, resulted in increased levels of tryptophane catabolites in bronchoalveolar lavages, which decreased macrophage production of proinflammatory cytokines (156). In mice, intranasal administration of Lactobacilli was more potent in reducing allergic airway inflammation than intragastric administration, possibly linked to an increase in regulatory T lymphocytes in the lungs (159). Interestingly, Lactobacilli were found to be significantly less abundant in the lungs of preterm infants who develop BPD compared to preterm infants who are BPD resistant (152). Within this cohort, Lactobacilli abundance was particularly low in infants born to mothers with chorioamnionitis. Coincidentally, azithromycin treatment could reduce the risk of BPD in preterm infants (160), particularly those colonized with *Ureaplasma* spp., which have been associated with chorioamnionitis and BPD (161, 162). The beneficial impact of the lung microbiome and specifically Lactobacilli on lung development is supported by a study in mice, where there was a positive correlation between microbial abundance and lung development (163). Injection of Lactobacilli into the lungs of germ-free mice could improve alveolar development (163).

Potential Therapies

In the near future, a potentially interesting avenue of therapy for the prevention or treatment of BPD may be the further exploration of the benefits of azithromycin. Following the bacterial lung microbiome, the lung virome and mycobiome are now slowly also becoming unraveled, which may provide further insights and treatment opportunities (148, 164, 165). Additionally, the benefits of pre- or probiotics to promote a healthy growth promoting lung microbiome should be investigated, and in particular the presence of Lactobacilli. D-Tryptophane was recently identified as a potent probiotic that could ameliorate allergic airway inflammation in a mouse model of allergic airway disease, and may therefore also be of interest in the setting of BPD (166). One possible way to achieve the same effect as tryptophane catabolites may be through the proton pump inhibitor omeprazole, which induces detoxification enzyme cytochrome P540 (CYP)1A1 possibly through an AhR-mediated process (167). AhR signaling is protective against hyperoxic injury in human fetal pulmonary microvascular cells and neonatal mice, likely because of its potent effects on the gene expression of immunomodulatory and developmental pathways (168). Combined pre- and postnatal omeprazole administration could attenuate hyperoxic lung injury in preterm rabbits even at low doses, making omeprazole an interesting potential therapeutic intervention to prevent BPD (167). Further studies are needed to validate its effects and to ascertain that it has no adverse effects on other developing organs.

Anti-inflammatory Agents

Bronchopulmonary dysplasia is primarily considered to be a developmental disease resulting from perinatal inflammation, and therefore specialists in the field have for the past decade called for a special focus on the development and improvement of anti-inflammatory therapies in BPD (169). Currently there are multiple anti-inflammatory therapies under investigation. Interleukin 1 receptor antagonist (IL1RA) is particularly promising, as it can prevent the development of experimental BPD when administered at a low dose in the neonatal rodent "double hit" model of BPD, consisting of hyperoxia and perinatal inflammation (170–172). In a sheep model for prenatal inflammation, intra-amniotic IL1RA could partially prevent the effects that lipopolysaccharide (LPS) had on lung maturation, measured as

surfactant protein gene expression and lung compliance (173). Interestingly, preterm infants who go on to develop BPD have elevated levels of IL1RA in their tracheal aspirates (174). A more recent study in preterm ventilated baboon and human infants suggested however that an increased IL1β:IL1ra ratio on days 1 to 3 of life is more predictive of BPD (172). The same study provided compelling animal data that early IL1RA or glyburide therapy, which prevents the formation of the NLR family, pyrin domain containing 3 (NLRP3) inflammasome upstream of IL1β, can indeed ameliorate BPD development (172). IL1RA, also called anakinra or Kineret, and glyburide, also known as Diabeta, are both already approved by the Federal Drug Administration (FDA) for treatment in rheumatoid arthritis and type 2 diabetes, respectively, making them attractive treatment options. Future studies will have to show whether their use would also be safe in the neonatal setting.

Postnatal use of corticosteroids such as dexamethasone and hydrocortisone, which are potent anti-inflammatory compounds, can effectively reduce the incidence of BPD (175, 176). Despite this positive effect, there are significant adverse effects associated with systemic administration of corticosteroids. Short-term adverse effects include intestinal perforation, gastrointestinal bleeding, hypertension, hypertrophic cardiomyopathy, hyperglycemia, and growth failure, while follow-up studies pointed to adverse effects on neuronal development (175, 176). Experts in the field have therefore questioned whether the beneficial effects of reducing BPD and death can be weighed up to these significant adverse effects (175, 176), and are reluctant to recommend postnatal systemic corticosteroids for the prevention of BPD (177). A perhaps more compelling alternative would be to more specifically target the lung through intratracheal administration. Early results obtained with inhaled corticosteroids have been mixed (178, 179), likely due to its efficiency to reach the lung parenchyma. However, as more studies are being done, there is increasing evidence that inhaled corticosteroids prevent BPD and death when administered early, but long term follow-up studies are needed to assess the risk-benefit ratio (180–182). Recent *in vitro* studies in human fetal lungs attributed budenoside more potent anti-inflammatory effects than dexamethasone, swiftly decreasing gene expression of chemokines IL8 and CCL2 (MCP1) in whole lungs even in the presence of exogenous surfactant (183). Future validation studies should however closely monitor the combined effect of intratracheal corticosteroids and pre-existing pulmonary inflammation, as combined antenatal exposure of fetal sheep to LPS and corticosteroids had much stronger effects on lung inflammation and developmental pathways than either agent alone (184–187). Additionally, it will be important to validate with combined budenoside and surfactant treatment also has the potential to prevent BPD in premature infants that initially present with mild RDS and do not receive surfactant therapy (188).

Potential Therapies

As outlined above, IL1RA, glyburide, and inhaled budenoside are currently the most promising anti-inflammatory therapies that have the potential to prevent BPD in premature infants. However, more studies will have to look into the safety and potential long-term effects in human neonates.

Reactive Oxygen Species (ROS) and Mitochondrial Dysfunction

Although BPD pathogenesis has a very multifactorial nature, with oxygen exposure, mechanical ventilation and inflammation as some of the most widely accepted causes, one common pathway is shared by these insults: the generation of ROS. In animal models, exposure of neonatal animals to hyperoxia within a specific time period is sufficient to induce a pathophysiology similar to BPD (189). Underlying this pathophysiology is an exaggerated mitochondrial oxidant stress in response in newborn mice compared to adults, with an overall lower expression of antioxidant enzymes (190). The response to hyperoxia is developmentally regulated, leading specifically to the production of mitochondrial ROS-dependent NADPH oxidase 1 (NOX1) expression in neonatal animals (191). Expression of antioxidant enzymes is controlled by AhR, as AhR-deficient fetal human pulmonary microvascular cells displayed significantly attenuated antioxidant enzyme expression and increased hyperoxic injury (192). Deficiency of another key antioxidant enzyme, extracellular superoxide dismutase (EC-SOD), was sufficient to impair alveolar development and induce pulmonary hypertension in mice (193). This phenotype was worsened by additional oxidative stress caused by bleomycin exposure, which was also associated with decreased VEGF signaling (193). Further support for the hypothesis that ROS formation also plays a role in human BPD development has come from a genetic study in very low birth weight infants, which found an association between single nucleotide polymorphisms (SNPs) in antioxidant response genes and an increased or decreased risk for the development of BPD (194). The role of antioxidant enzymes in neonatal chronic lung disease is reviewed in depth by Berkelhamer and Farrow (195).

Mitochondria play a central role in oxygen metabolism, and mitochondrial abundance as measured by mitochondrial protein expression peaks around birth to facilitate the transition to the oxygen-rich world outside the womb (196, 197). Preterm infants are born before this peak, making them less prepared to deal with this shift in bioenergetics. Besides this mitochondrial immaturity, the exposures leading to chronic lung diseases have been linked to mitochondrial dysfunction (198, 199). Both hyperoxia exposure and mechanical ventilation of neonatal mice caused pulmonary mitochondrial dysfunction (200, 201). Moreover, direct inhibition of mitochondrial oxidative phosphorylation significantly impaired alveolar development, comparable to hyperoxia or mechanical ventilation. *In vitro* experiments indicate that elevated CO_2 levels, called hypercapnia, a common occurrence in BPD patients, also causes mitochondrial dysfunction (202). One potential mechanism through which mitochondrial dysfunction and ROS generation potentially lead to impaired alveolar development in hyperoxia exposed neonatal mice is through endoplasmic reticulum (ER) stress, which can cause apoptosis (82).

Potential Therapies

In animal studies, several potential treatments have been identified to decrease ROS generation. In neonatal mice, treatment with a specific mitochondrial antioxidant, (2-(2,2,6,6-tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl)triphenylphosphonium chloride (mitoTEMPO), could protect against hyperoxia-induced

lung injury (191). Another promising treatment compound is GYY4137, a slow-releasing H_2S donor, which could decrease ROS generation and thus protect and restore normal alveolar and microvascular development after neonatal hyperoxia injury in rats (203). Targeting the AhR would appear to be another promising approach considering it also has potent anti-inflammatory properties, as described above. Although omeprazole is generally seen as a potentiator of AhR activation, omeprazole treatment of hyperoxia-exposed newborn mice counterintuitively decreased functional AhR activation, worsening hyperoxic injury (204). Other approaches to promote AhR activation may however prove to be more effective. An entirely different approach in treating mitochondrial dysfunction may be through mitochondrial transfer, a process that has been reported as one of the therapeutic mechanisms of MSC therapy (205). In human BPD patients, most neonatal antioxidant trials have unfortunately not shown any benefit in the prevention of BPD, with the exception of vitamin A therapy (195). However, none of these antioxidant therapies were specifically targeted against mitochondrial ROS or dysfunction. More targeted approaches, as those outlined in the animal studies, may prove to be more promising.

Other Promising Therapeutic Options Based on Novel Pathophysiological Insights

Inflammation associated with BPD pathogenesis affects many molecular pathways, which by themselves can be interesting therapeutic targets. One of these is the ceramide pathway, which is upregulated in both hyperoxia and antenatal inflammation animal models (206–208) and also in other chronic lung diseases such as asthma, cystic fibrosis and COPD (209). Increased ceramide levels lead to increased apoptosis, both in epithelial cells of BPD patients and in animal models of BPD (208, 209). Intervention with a sphingosine-1-phosphate (S1P) analog in the mouse hyperoxia model of BPD could successfully ameliorate ceramide levels and hyperoxia-induced alveolar hypoplasia (208). In a more complex piglet model of lung injury by lavage, LPS instillation and injurious ventilation, tracheal installation with surfactant and D-myo-inositol-1,2,6-trisphosphate (IP3) could achieve a similar effect in reducing ceramide levels and improving oxygenation (210). In a different approach to decrease sensitivity to apoptosis in hyperoxia-exposed epithelial cells, inhibiting regulatory-associated protein of mechanistic target of rapamycin (RPTOR) could prevent hyperoxia-induced lung injury in neonatal mice (211). Based on these studies, selective pharmacological interventions which temporarily reduce apoptosis could be a promising way to prevent or repair neonatal lung injury and reduce BPD severity.

An intervention that has garnered attention in neonatal care is lactoferrin (LF), an iron-binding protein that is a normal component of human colostrum and milk (212). It has potent antimicrobial activity, can stimulate the innate immune system and promote epithelial proliferation and differentiation of the immature gut (213). Recent studies have identified LF supplementation as a promising agent for the reduction of late onset sepsis and necrotizing enterocolitis (214). Although the properties of LF may also be desirable for the prevention of BPD, to date no study has been able to show a significant reduction in the development of BPD following LF supplementation (214).

A pathophysiological mechanism of BPD that is slowly gaining more attention is the link between pre-eclampsia and BPD. Pre-eclampsia a proven risk factor for BPD (41), and the underlying impact on the developing fetus may be three-fold. Firstly, maternal preeclampsia is a frequent cause of preterm birth before 28 weeks (215). Secondly, severe preeclampsia can lead to intrauterine growth restriction, which in itself is a strong risk factor for BPD (38, 39). Thirdly, the placental dysfunction that lies at the root of pre-eclampsia leads to an overproduction of soluble VEGF receptor 1 [also known as soluble fms-like tyrosine kinase-1 (sFlt-1)], which inhibits VEGF signaling (216, 217). This not only leads to increased sVEGFR-1 in maternal serum, but also in amniotic fluid (218). By giving pregnant rats intra-amniotic injections with sVEGFR-1, Steven Abman's group demonstrated a link between pre-eclampsia and BPD, as neonatal rats presented with impaired alveolar and microvascular development and right and left ventricular hypertrophy (40). Moreover, intrauterine exposure to excess sVEGFR-1 led to increased apoptosis of endothelial and mesenchymal cells in neonatal rat lungs. Placental dysfunction and subsequent overexpression of sVEGFR-1 may therefore be a potential therapeutic target to improve fetal outcome and prevent development of BPD. At the very least, the diagnosis of maternal pre-eclampsia should be considered as a serious predisposition for the development of BPD.

From a developmental biology perspective, developmental molecular pathways that are downregulated in BPD provide other potential targets for the amelioration of BPD pathogenesis. These include the Wnt signaling pathway (187, 219, 220), SHH signaling (185, 221–223), axonal guidance cues semaphorin 3 C and ephrin B2 (224, 225), Notch signaling (226, 227), and HIFs (228). In addition, a wealth of new molecular insights on mouse and human lung development has been and will be published in the upcoming years by the LungMAP consortium (1U01HL122638), funded by the National Heart, Lung, and Blood Institute (NHLBI) (http://www.lungmap.net) (229, 230). BPD is generally considered to be caused by environmental factors, but in recent years studies have uncovered that a genetic component may also be at play [reviewed in Ref (231, 232)]. Although associations are not conclusive, these studies suggest that genetic variants of genes in well-known lung development and repair pathways may predispose for severe BPD or mild/moderate BPD (232). microRNAs have emerged as both a pathophysiological mechanism and a tempting tool to target transcription of multiple of these developmental signaling pathways at once. Although multiple human and animal studies have reported an association between altered microRNA levels and BPD, valid concerns have been raised about the lack of a causal link between altered microRNA levels and BPD pathogenesis [reviewed in Ref (233)]. However, if such a causal link can be confirmed, as was recently seen in a study which demonstrated the regulation of alveolar septation by microRNA-489 (234), the use of specific microRNA antagonists or agonists may be considered as a potential therapy for BPD. Caution should however be exercised when directly modulating potent developmental pathways, either directly or through microRNA therapy. Further exploration of such therapeutic targets

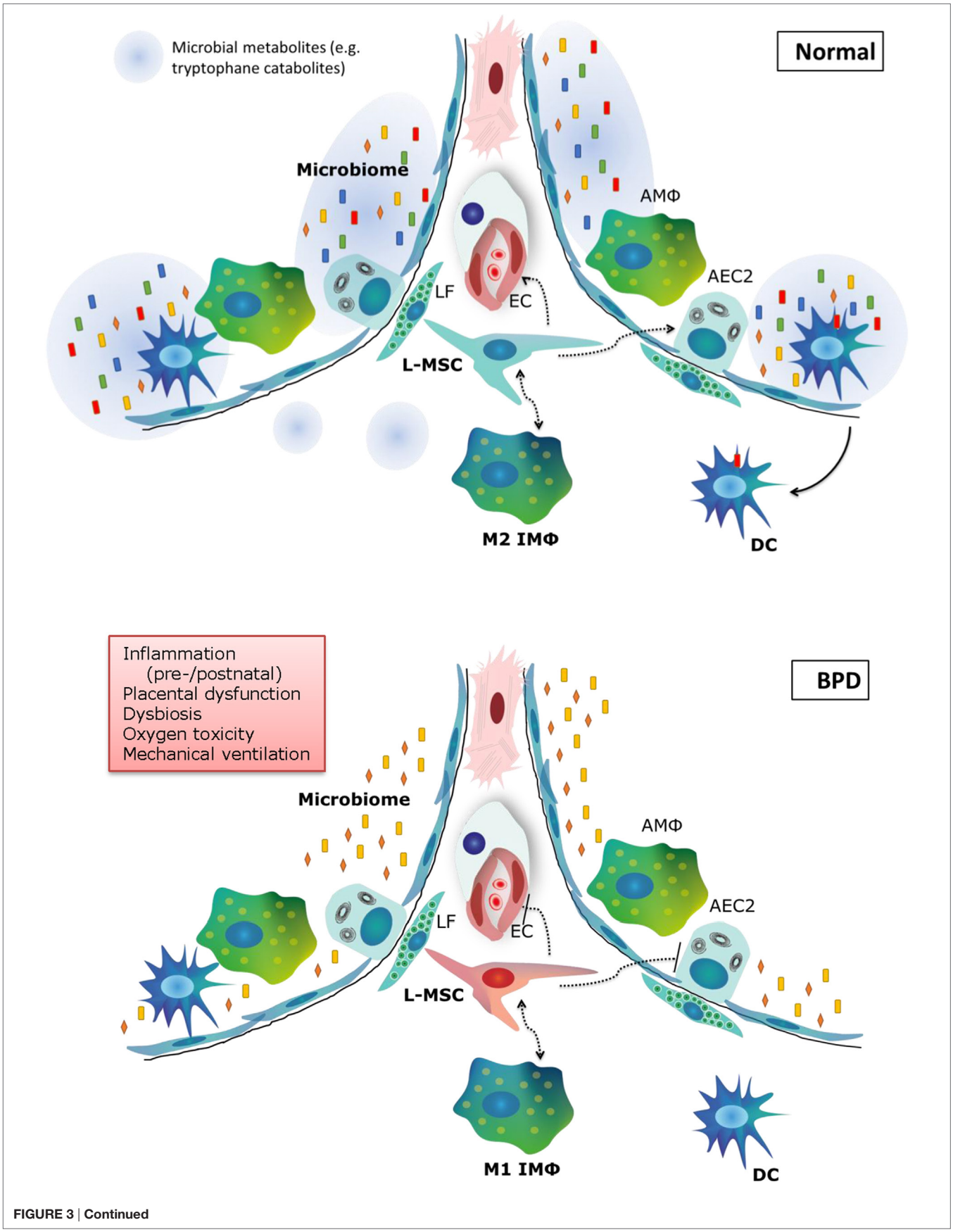

FIGURE 3 | **Continued**

FIGURE 3 | Continued
Summary of new pathophysiological concepts in bronchopulmonary dysplasia (BPD). In normal alveolar lung development, a diverse microbiome is necessary to train the pulmonary immune system and secrete metabolites that support lung development. Pulmonary M2 interstitial macrophages (M2 IMφ) are present and likely play an active role in lung development. L-MSCs support M2 IMφ, alveolar epithelial cells and the microvasculature. In BPD (bottom panel), pre- and postnatal risk factors lead to decreased microbiome diversity, a proinflammatory environment, dysfunctional L-MSCs, epithelial and endothelial injury and impaired repair. LF, lipofibroblast; EC, endothelial cell; AMΦ, alveolar macrophage; L-MSC, lung mesenchymal stromal cell; AEC2, alveolar epithelial cell type 2; M1/M2 IMΦ, type 1/2 interstitial macrophage; DC, dendritic cell.

should perhaps be combined with slow releasing microparticles or capsules to ensure a more physiological release and prevent pathological side effects.

Conclusion and Future Directions

The pathophysiology of BPD is extremely multifactorial, which is underlined by the emerging role of cell types that have only recently been acknowledged, such as the microbiome, macrophages, and tissue stem cells (**Figure 3**). Our knowledge on the pathophysiology is poised to move forward rapidly in the next decade, due to exciting new technological advances in the research field, and is opening avenues for the pursuit of therapeutic options. In addition, there is still promise for new and better applications of existing therapies, which have not yet fulfilled their promise in a clinical setting. In the next decade of BPD research, the most promising therapies and pathophysiological concepts that should be pursued for new therapeutic options are as follows:

- Animal models investigating the pathogenesis of BPD should identify different sub-pathophysiological processes that arise because of different combinations of pre-and postnatal exposures (e.g. pre-eclampsia, dysbiosis), as opposed to only looking at hyperoxia or inflammation models. Moreover, better appreciation of extrapulmonary issues related to BPD might be instructive, particularly neurodevelopmental outcome and retinopathy, which are frequent long-term outcomes resulting from BPD (235).
- Different routes of administration for effective therapies such as vitamin A and postnatal corticosteroids, in particular non-invasive intratracheal routes.
- Cell-based therapies, either through administration of stem cells and their products or by promoting the regenerative potential of resident lung stem cells.
- The commensal role of the pre- and postnatal (lung) microbiome in the normal and perturbed lung development, and its potential as a therapeutic target.
- The role of placental dysfunction in the pathogenesis of BPD, and its potential as a therapeutic target in the prevention of BPD.
- The role of the immune system not only as an adverse factor in BPD pathogenesis, but its importance in supporting normal lung development and repair.

AUTHOR CONTRIBUTIONS

Conception and outline of review: JC, DT, and RR. Writing of the manuscript: JC. Drafting of the manuscript: JC, DT, and RR. Critical revision of manuscript: JC, DT, IK, IR, and RR. Final approval of manuscript: JC, DT, IK, IR, and RR.

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REVIEW
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Imaging Bronchopulmonary Dysplasia—A Multimodality Update

*Thomas Semple[1,2], Mohammed R. Akhtar[3] and Catherine M. Owens[2]**

[1] The Royal Brompton Hospital, London, United Kingdon, [2] Great Ormond Street Hospital, London, United Kingdom, [3] St Bartholomews and The Royal London Hospital, London, United Kingdom

Bronchopulmonary dysplasia is the most common form of infantile chronic lung disease and results in significant health-care expenditure. The roles of chest radiography and computed tomography (CT) are well documented but numerous recent advances in imaging technology have paved the way for newer imaging techniques including structural pulmonary assessment *via* lung magnetic resonance imaging (MRI), functional assessment *via* ventilation, and perfusion MRI and quantitative imaging techniques using both CT and MRI. New applications for ultrasound have also been suggested. With the increasing array of complex technologies available, it is becoming increasingly important to have a deeper knowledge of the technological advances of the past 5–10 years and particularly the limitations of some newer techniques currently undergoing intense research. This review article aims to cover the most salient advances relevant to BPD imaging, particularly advances within CT technology, postprocessing and quantitative CT; structural MRI assessment, ventilation and perfusion imaging using gas contrast agents and Fourier decomposition techniques and lung ultrasound.

Keywords: bronchopulmonary dysplasia, structural characterization, imaging techniques, quantitative pulmonary magnetic resonance imaging, lung parenchymal magnetic resonance imaging, hyperpolarized gas imaging, lung ultrasound

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****Correspondence:***
Catherine M. Owens
owenscatherine.5@gmail.com

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BRONCHOPULMONARY DYSPLASIA (BPD)

Bronchopulmonary dysplasia is the most common form of infantile chronic lung disease and is reported to occur in between 10.2 and 24.8% of European infants born between 24 + 0 and 31 + 6 weeks of gestation (1). While only representing 8% of births in population-based data from the US, preterm or low-birth weight infants accounted for 47% of the total annual expenditure for all births (2, 3).

The clinical definition of BPD is the requirement of supplemental oxygen for at least 28 days in an infant born at less than 32 weeks of gestation (4).

The classic form of BPD was described in premature infants exposed to prolonged high-pressure mechanical ventilation and high concentrations of inspired oxygen (5). Pathological findings include alternating regions of overinflation and atelectasis, airway smooth muscle hypertrophy, squamous metaplasia of the airway epithelium, peribronchial fibrosis, constrictive obliterative bronchiolitis, and hypertensive pulmonary vascular changes (6).

While the current widespread administration of antenatal steroids, adoption of lower pressure ventilatory support, and reduction in the use of high concentration inspired oxygen have led to a decreased incidence of classical BPD, the increased survival of extremely premature (24–26 weeks of gestation) low-birth weight (<1,000 g) neonates has produced a new variant of BPD (7). Extremely

premature neonates tend to respond well to the administration of exogenous surfactant and require relatively low-pressure mechanical ventilation with low to moderate oxygen concentrations. However, they are more prone to infection and pulmonary edema from physiological shunts (e.g., patent ductus arteriosus) leading to increased respiratory support needs (8). The lungs of neonates born at 24–28 weeks of gestation are still undergoing significant development and maturation, transitioning from the canalicular stage (formation of acina and invasion of capillaries into the pulmonary mesenchyme), through the saccular stage (formation of alveolar saccules from the terminal bronchioles) toward the alveolar phase at around 32 weeks of gestation, where the first true alveoli are formed (9). Birth and premature initiation of gas exchange will interrupt this development, with studies demonstrating the presence of fewer, larger (simplified) alveoli with reduced vascularity in the lungs of neonates born prematurely (10, 11). Pathologic specimens demonstrate a lower incidence of airway and vascular diseases and less interstitial fibrosis than in the more severe classic form of BPD (12).

In the longer term, alongside other disorders related to prematurity, BPD can lead to recurrent hospitalizations with lower respiratory tract infections, reduced lung function, severe obstructive airways disease, and pulmonary hypertension with right heart dysfunction (13, 14) with neurological and cognitive impairment causing further morbidity (15). Interestingly, a recent study has suggested an association with pulmonary vein stenosis (PVS) with 4.6% of a 213-patient cohort of infants with BPD affected, more frequently those with lower birth weights. Those with associated PVS experienced higher rates of mortality (16).

ROLE OF IMAGING IN BPD

During their initial neonatal intensive care unit (NICU) admission the imaging modality most commonly utilized in premature infants is chest radiography, allowing simultaneous assessment of support apparatus (endotracheal tubes, umbilical arterial, venous catheters, etc.), pulmonary parenchymal status [degree of respiratory distress syndrome (RDS) related change, edema from persistent shunting *via* a patent ductus arteriosus, etc.], and complications of mechanical ventilation (pneumothorax, pulmonary interstitial emphysema, etc.).

Chest radiographic features of established BPD include interstitial thickening, focal or generalized hyperexpansion, and atelectasis (**Figure 1**) (17). Computed tomography (CT) is more sensitive to the abnormalities of BPD demonstrating abnormalities in over 85% of patients with BPD including regions of decreased attenuation, emphysema-like change, linear and subpleural opacities, and bronchial wall thickening (**Figure 2**). Furthermore, the extent of structural abnormality on CT has been shown to correlate with the clinical severity of BPD (18).

There have been many developments within pediatric thoracic imaging (and indeed medical imaging in general) over the past 5–10 years with great potential to shed further light on the pathogenesis and temporal evolution of respiratory conditions such as BPD, further guide the treatment of RDS with the goal of reducing the subsequent development of BPD, and in the long-term follow-up of chronic respiratory disease. Some of the more significant developments are discussed below.

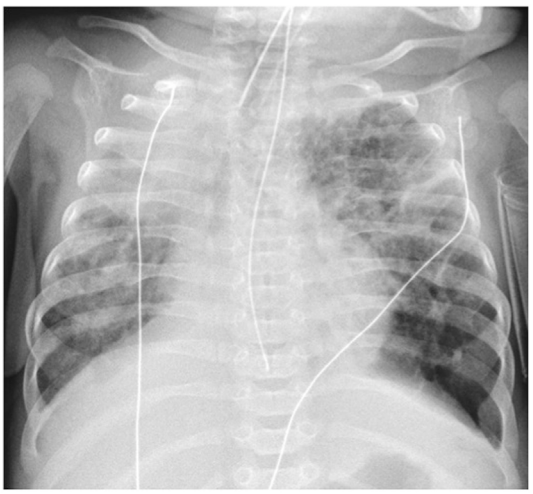

FIGURE 1 | Chest radiograph demonstrating widespread coarse interstitial markings, atelectasis, and regions of hyperexpansion (particularly at the left lung base), typical of bronchopulmonary dysplasia. Note also the right upper lobe consolidation and malposition of the NG tube.

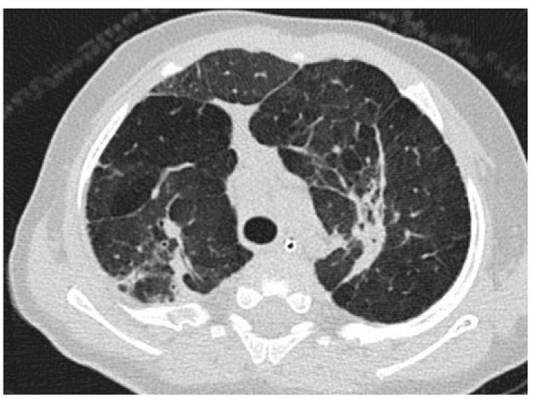

FIGURE 2 | Axial computed tomography section through the upper lobes on lung window settings demonstrates linear and subpleural opacities, bronchial wall thickening, and areas of low attenuation (indicative of small airways disease) in a patient with bronchopulmonary dysplasia.

EVOLUTION OF IMAGING TECHNIQUES RELEVANT TO BPD

Computed Tomography

Traditional "step and shoot" high-resolution computed tomography produced non-contiguous (interrupted) high spatial resolution images that could only be viewed in a single (axial) anatomical plane. This method has now largely been replaced with spiral/volumetric acquisitions that produce continuous volumetric data sets with isotropic voxels (each voxel—three-dimensional

pixel—is the same length in *x*, *y*, and *z* axis). This allows reconstruction of the data in any plane (multiplanar reconstruction) and is essential for the more advanced postprocessing techniques discussed below.

Recent advances in CT technology have resulted in faster (subsecond) CT X-ray tube rotation speeds and smaller, more sensitive radiation detectors resulting in significant improvements in both temporal and spatial resolution (19). Current state of the art CT scanners are available with a single 320-row detector array, allowing the coverage of 16 cm in the *z*-axis (craniocaudal length) in a single tube rotation. An alternative arrangement (dual source CT) consists of two X-ray tubes (rather than the traditional single tube) with two arrays of detector banks mounted at 95° to each other such that two interlocking spiral data sets are formed around the patient, thus scanning the same volume of tissue in half the time as a single source scanner. Both these methods allow an infant's entire chest to be imaged in a fraction of a second (19, 20). This combination of faster tube rotation speeds, greater numbers of detectors and dual source systems, alongside the use of immobilization devices, such as vacuum splints (**Figure 3**) to keep the child still reducing the effects of patient body movement, has resulted in a paradigm shift within pediatric chest imaging, from scans requiring general anesthetic and breath-holding maneuvers, to ultrafast scans that produce diagnostic quality images, with minimal respiratory and cardiac motion artifact without the need for light sedation (21). Even at the high heart rates typical within the neonatal population, high-pitch CT, following the administration of intravenous (IV) contrast material has been proven capable of demonstrating small, fast-moving structures, such as the pulmonary veins, in diagnostically acceptable detail (22).

It is well known that the radiation burden of conventional thoracic CT is greater than that of chest radiography; however, technological advances (including rotational tube current modulation, adaptive array detectors and the introduction of iterative reconstruction techniques), alongside departmental dose optimization programs, have resulted in a significant reduction in CT radiation dose, while maintaining and even improving diagnostic image quality. There is also work in progress regarding the feasibility of ultralow dose thoracic CT with equivalent doses of the same order as chest radiography. Shi et al demonstrated a drop in equivalent dose from 0.89 to 0.61 mSv with no statistically significant difference in perceived image quality, and only a 14.8% decrease in measured signal to noise ratio when reducing tube voltage from 80 to 70 kV (23).

Postprocessing techniques are playing an increasingly important role within cardiothoracic imaging. Basic reconstruction into multiple orthogonal planes allows for easy differentiation of pulmonary vessels from parenchymal nodules. Increasing the slice thickness (average intensity projection) can reduce image noise in low dose examinations of small infants. Maximum and minimum intensity projection images (MIP and MinIP, respectively) can be utilized to better demonstrate vasculature and low attenuation regions such as regions of air trapping, respectively (24, 25). MinIP images are particularly well suited to demonstrating the areas of low attenuation alternating with higher attenuation lung (variegate mosaic attenuation) seen in patients with a small airways component of BPD (**Figure 4**).

More advanced postprocessing techniques such as volume rendering techniques allow the formation of 3D images of lungs and airways that can aid discussion with the wider multidisciplinary respiratory team and with families in clinic. There is further on-going research into the possible role of quantitative CT measures of lung volume, assessment of bronchial wall thickness, and quantification of abnormally low attenuation lung allowing potentially more robust and reproducible measures of airway and lung parenchymal disease (26).

As quantitative CT measures of respiratory tract disease become more mainstream, the necessity for protocol standardization will become more important, particularly in young infants who cannot follow breathing instructions, resulting in scan acquisitions during variable phases of the respiratory cycle. Attempts

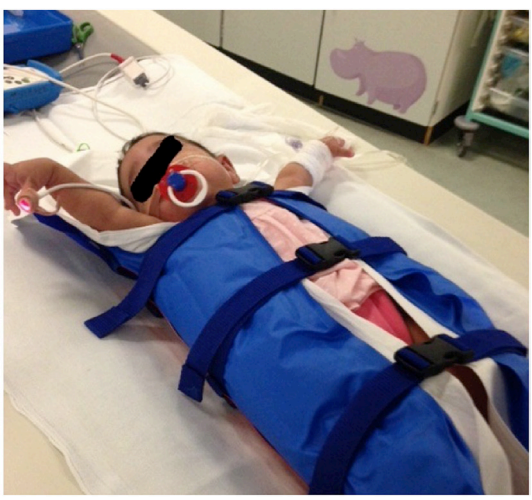

FIGURE 3 | Vacuum immobilization device used to limit gross patient movement. Use of these devices, alongside ultrafast, high-pitch computed tomography, has dramatically reduced the need for general anesthetic or sedation for cardiothoracic CT at our institution.

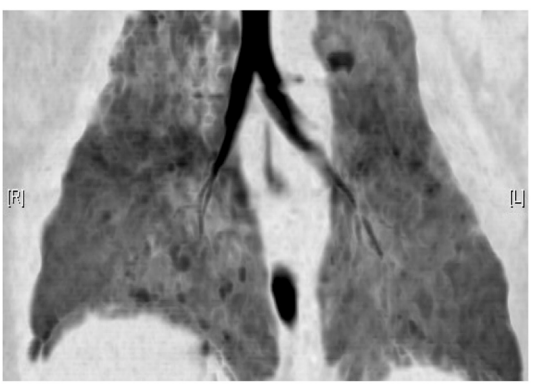

FIGURE 4 | Minimum intensity projection CT reconstruction demonstrating airway morphology and regions of heterogeneous (mosaic) attenuation in a child with bronchopulmonary dysplasia.

to overcome this problem, including spirometer-triggered CT, are currently in use in several specialist centers (27, 28).

Magnetic Resonance Imaging (MRI)

As a cross-sectional imaging technique that does not rely on ionizing radiation exposure, MRI seems the ideal modality for cross-sectional imaging in the pediatric population. However, the significant inherent limitations of conventional MRI severely limit its use in pediatric thoracic imaging. The lung parenchyma is inherently low in proton density and contains many air–tissue interfaces. As such, it returns extremely low levels of rapidly decaying signal, resulting in the formation of extremely low-resolution images of the lung parenchyma and all tissues save for the most central airways. Long examination times necessitate general anesthesia or heavy sedation and produce significant respiratory and cardiac motion artifacts (29). Further limitations result from the overall large size and reasonably small inner bore of the scanner, the need to transfer an unwell infant from the NICU to the MRI scanner, and the magnetic field strength which limits the level of medical support an infant can be provided without the use of specific MRI safe monitoring and anesthetic equipment.

More robust respiratory and ECG/pulse gating techniques, along with new RF pulse sequences, and sampling and reconstruction techniques have significantly improved the visualization of the pulmonary parenchyma and airways resulting in renewed interest in structural lung assessment *via* MRI. Faster MRI sequences such as T2-HASTE (single shot half-Fourier turbo spin echo) and T1 3D gradient recalled echo with parallel imaging algorithms (e.g., generalized autocalibrating partially parallel acquisition) have been employed with more recent interest in radial acquisitions [e.g., Periodically Rotated Overlapping ParallEL Lines with Enhanced Reconstruction (PROPELLER)], which are less sensitive to respiratory motion artifact, and ultrashort echotime sequences such as pointwise encoding time reduction with radial acquisition (PETRA)—a noiseless, free breathing sequence capable of isometric data acquisition at a submillimeter voxel size (30–32).

While significant headway has been made in improving structural lung assessment *via* MRI, the spatial resolution remains poor relative to CT (**Figures 5A,B**) [PETRA achieved a voxel size of 0.86 mm^3 compared to 0.2 mm^3 from a state of the art CT scanner (19)] and image acquisition time remains high [8–12 min for PETRA (33), 7–10 min for respiratory triggered PROPELLER (31) compared to a fraction of a second *via* CT]. Lung MRI may, however, have far more to offer in terms of quantitative and functional data output. Multiple acquisitions following the administration of IV contrast material (gadolinium chelate) allow the study of regional pulmonary perfusion over time (**Figure 6**). Newer techniques allow the formation of similar perfusion "maps" without the administration of contrast media and the associated risk in the presence of renal dysfunction (particularly relevant in premature infants). A variant of arterial spin labeling (ASL-FAIRER arterial spin labeling-flow sensitive alternating inversion recovery with an extra radiofrequency pulse) techniques involving the use of magnetic "tagging" of inflowing blood as a contrast medium has been used to study regional pulmonary perfusion without the need for IV administration of contrast medium (32). A second mathematically derived technique, Fourier decomposition, enables the formation of both perfusion and ventilation maps, again without the need for IV contrast administration, by extracting (decomposing) signal acquired throughout the respiratory cycle at respiratory and pulse frequencies, and has been shown to be feasible in children with cystic fibrosis (34).

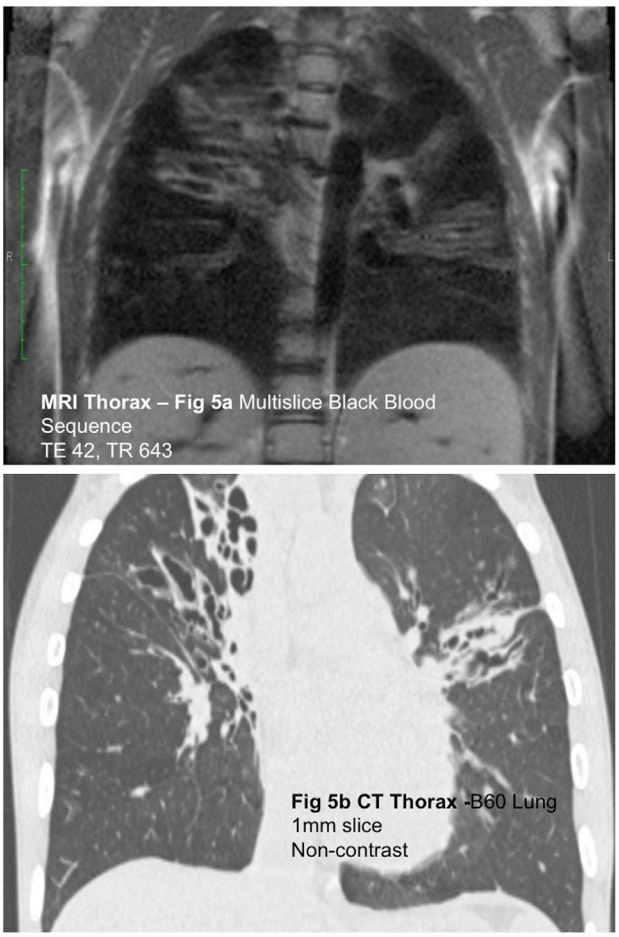

FIGURE 5 | **(A)** Coronal black blood SSFP magnetic resonance (MR) image and **(B)** coronal computed tomography (CT) reconstruction in a child with cystic fibrosis. Although the spatial resolution of MRI is relatively poor compared to CT, MRI is capable of demonstrating gross airway pathology.

Ventilation imaging *via* MRI has been extensively investigated with the highest resolution images obtained *via* the administration of hyperpolarized noble gases (typically He^3 or Xe^{129}) (35). Similar direct imaging of ventilation is also possible through the inhalation of fluorinated gases (e.g., sulfur hexafluoride and hexafluoroethane) (36). It is also possible to measure the degree of diffusion of these gases using multiple rapidly acquired diffusion-weighted images at differing B-values to provide a "short range" apparent diffusion coefficient (37). The free diffusivity of ^3He makes it ideal for ventilation imaging, but the solubility of ^{129}Xe and oxygen allows imaging of not only the inhalational phase but

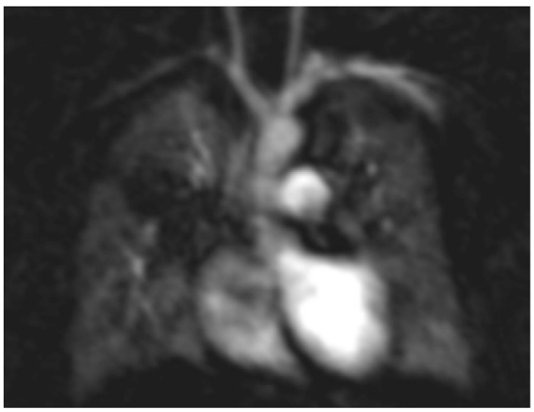

FIGURE 6 | Magnetic resonance imaging angiogram in a child with bronchopulmonary dysplasia demonstrating poor perfusion of the right upper lobe related to severe small airways disease and reflex vasoconstriction.

also the tissue and blood phases, giving further potentially useful information regarding the whole gas-exchange process (38).

Oxygen imparts a concentration-dependent paramagnetic effect on the rate of T1 recovery in adjacent tissue. Rapid T1 mapping *via* low flip angle GRE or "FLASH" (fast low angle shot) sequences before and at multiple concentrations of inhaled oxygen can thence produce an imaging measure of oxygen transfer (the oxygen transfer function—OTF) (39). The ready availability of oxygen as a medical gas and lack of the need for expensive hyperpolarization equipment make this a particularly attractive option for MR ventilation imaging. A combination of inversion pulses and single shot fast spin echo sequences, with prospective respiratory gating and retrospective deformable image registration, interleaved 2D slices with parallel imaging and half-Fourier reconstruction, allows whole lung oxygen-enhanced imaging of adult patients within 8–13 min (40).

While many of the abovementioned methods of ventilation/perfusion MRI have yet to be reported in the context of BPD, the development of a small footprint 1.5 T MRI unit installed on the neonatal unit of Cincinnati Children's Hospital has allowed several studies of MRI utilization in the investigation of neonatal lung disease. One such study identified a significantly higher volume of "high signal lung" in infants with BPD than was demonstrated in premature infants without BPD and healthy term infants. However, it should be noted that small numbers of infants were included (six term, six premature without BPD, and six infants with BPD) and that the infants with BPD were significantly lower weight and gestational age than the premature non-BPD and term groups. Also that "high signal" was defined as signal over 45% of the patient's mean chest wall signal without any mention of differing muscle mass/fat composition between groups. The study also assumes that the T1, T2, and T2* relaxation times of lung parenchyma and chest wall soft tissues are identical. While quantitative measurements of MRI signal in small neonates are in their infancy and should be interpreted with caution, this group did produce diagnostic-quality cross-sectional images of the lung parenchyma with no general anesthetic or sedation, with infants scanned during a 1.5-h period of free breathing. Two infants with BPD also underwent CT. In comparison with 3 mm CT sections (as opposed to more conventional 1 mm sections), CT demonstrated a greater number of regions of hyperlucent "emphysema-like" change and more severe bronchovascular distortion than MRI (Ochiai structural BPD score *via* CT of 12 vs 9 *via* MRI) (41).

Clearly significant headway has been made in pulmonary MRI, both in terms of structural and quantitative/functional imaging capability; however, further work, particularly regarding reproducibility and the clinical significance of quantitative/functional measures, remains to be done, before MRI can become a part of routine clinical care.

Ultrasound

Studies have suggested a role for ultrasound in the assessment of premature neonates with RDS (also known as hyaline membrane disease—HMD) in predicting the development of BPD. Avni et al. reported homogeneous hyperechogenicity of the lung bases, obscuring the diaphragm on transhepatic/transsplenic ultrasound in the setting of HMD with hyperechoic reverberation artifacts, beyond that expected at the diaphragmatic position. This "HMD-pattern" was found to transform to a "BPD pattern" of streaky, irregular areas of lower echogenicity, seen at day 18 of life in all patients subsequently diagnosed with BPD, with a negative predictive value of 95% (42). A further study by Pieper et al. demonstrated similar changes with the greatest predictive value of subsequent BPD diagnosis achieved *via* ultrasound at day 9 of life. They did, however, also observe a false positive case with the "BPD pattern" caused by bilateral lower lobe pneumonia (as demonstrated *via* chest radiography) (43). Clearly, there may be a specific role of lung ultrasound in the prediction of the development of BPD, however, these appearances (essentially artifacts) cannot be interpreted in isolation, and overall ultrasound is not a safe alternative to chest radiography. Complications of mechanical ventilation such as the misplacement of tubes and lines, air leaks (pneumothorax, pneumomediastinum, and pulmonary interstitial emphysema), and central pathology that do not abut the pleural surface can be completely invisible *via* ultrasound. There are, however potential roles in the setting of longitudinal research studies (as utilized in the Drakenstein Child Health Study), particularly in resource-poor areas (44).

CONCLUSION

Despite numerous significant advances within imaging technology, especially in CT and MRI, the simple chest radiograph remains the cornerstone of pediatric parenchymal lung imaging, particularly in the setting of premature neonates receiving complex support on a NICU. CT is reserved for specific clinical questions, including the presence of complex pathology and the more recently recognized association of prematurity with PVS.

New low and ultra-low dose CT techniques have brought the radiation exposure associated with CT closer to that of plain radiography and faster CT scanners have significantly reduced the need for general anesthetic and sedation use when imaging small children.

Improvements in respiratory and pulse gating in MRI alongside newer faster sequences and acceleration techniques have significantly improved the spatial resolution of pulmonary parenchymal MRI; however, the resolution remains inferior to that of CT. Coupled with long examination times, the role of MRI in pediatric pulmonary parenchymal imaging therefore remains predominantly as a research tool.

Ventilation MRI with hyperpolarized noble gases, fluorinated gases, oxygen, or *via* Fourier decomposition is making significant potential but again remains a research tool at this time.

Quantitative imaging by CT (lung volume calculation, bronchial wall thickness measurement, and low attenuation mapping) and MRI (OTF, quantification of regional signal) is showing significant promise, but still needs to be interpreted with care. It is clear that if imaging moves away from a traditional structural assessment toward a quantitative assessment, significant care will have to be taken to standardize examination techniques both within and between institutions. There is a very real risk that without a high level of standardization these techniques amount to a poor attempt at functional imaging, at a spatial resolution far below that of conventional nuclear medicine without its established robust clinical correlation.

Ultrasound has potentially established a niche use within risk assessment of premature neonates and may guide the future treatment of infants deemed to be at higher risk following the first few weeks of life. It should, however be noted that it does not constitute a potential replacement for plain radiography as suggested by some authors (45), as central pathology and important complications arising from misplaced support apparatus or air leaks can be completely missed *via* ultrasound alone.

Clearly, we are at an exciting crossroads between conventional structural and novel quantitative and functional imaging assessment, with ample room for new technology to significantly influence the future of neonatal pulmonary imaging. As progressively more sophisticated and complex technology is introduced it becomes increasingly important to stay up to date with advances and to maintain a detailed understanding of each technique. Novel techniques need validation within large cohorts of patients paying careful attention to protocol standardization. In the meantime, the humble chest radiograph is here to stay.

AUTHOR CONTRIBUTIONS

Imaging bronchopulmonary dysplasia—a multimodality update. All persons who meet authorship criteria are listed as authors, and all the authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Authorship contributions: Category 1, conception and design of study: TS, CO, MA; acquisition of data: TS, CO, MA; analysis and/or interpretation of data: TS, CO, MA; Category 2, drafting the manuscript: TS, CO, MA; revising the manuscript critically for important intellectual content: TS, CO, MA; category 3, approval of the version of the manuscript to be published (the names of all authors must be listed): TS, CO, MA.

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frontiers in Medicine

GENERAL COMMENTARY
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Commentary: Expert Opinion to "Imaging Bronchopulmonary Dysplasia—A Multimodality Update"

Mark O. Wielpütz [1,2,3]*

[1] Translational Lung Research Center (TLRC), German Center for Lung Research (DZL), University of Heidelberg, Heidelberg, Germany, [2] Department of Diagnostic and Interventional Radiology, University of Heidelberg, Heidelberg, Germany, [3] Department of Diagnostic and Interventional Radiology With Nuclear Medicine, Thoraxklinik at University of Heidelberg, Heidelberg, Germany

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A Commentary on

Imaging Bronchopulmonary Dysplasia—A Multimodality Update
by Semple, T., Akhtar M. R., and Owens, C. M. (2017). Front. Med. 4:00088. doi: 10.3389/fmed.2017.00088

Chronic lung disease in infancy and early childhood poses a diagnostic challenge to medicine. (1) Entities are rare, with bronchopulmonary dysplasia (BPD) being the most prevalent in infancy and with increasing prevalence in grown-ups due to improved survival of prematurity. (2) Functional impairment is hard to measure because spirometry is practically impossible to perform at an early age. (3) Structural imaging may be a key component to diagnosis and monitoring, with computed tomography delivering high spatially resolved morphological information, but cumulative radiation dose and periprocedural efforts may be of high concern in radiation-susceptible young individuals and restrictive for imaging procedures (1). (4) Further, apart from definite diagnosis, therapeutic relevance should guide the decision to perform imaging tests and must be weighed against potential risks. Obviously, this somewhat trivial dogma holds true for all diagnostic tests in pediatric and adult clinical medicine, but it is specifically important in the research context where the generation of advances in knowledge and the testing of hypotheses may require a more liberal—yet well-considered—acquisition of study data, even in studies of human subjects. In the case of pediatric lung disease, imaging with structural and functional techniques may be the most comprehensive approach to characterize a pathophysiological process of the lungs and to study the natural evolution of findings and the changes induced by therapeutic intervention. In the case of new medication made available, imaging may provide a sensitive endpoint for measuring successful treatment (2).

Cystic fibrosis lung disease has been the most prominent example, for which sustained efforts in avoidance of radiation and improving imaging diagnostics have lead to the stepwise introduction of proton magnetic resonance imaging (MRI) for structural and functional lung disease, with a widely available MRI protocol, a scoring system for semi-quantification (3), cross-validation against CT (4), X-ray (5, 6), multiple breath wash-out (7) and spirometry (8), initial studies using MRI to monitor mid-term natural evolution (8), and as an endpoint for therapeutic intervention (6, 7, 9, 10). Of note, contrast-enhanced perfusion MRI is the first among many functional techniques to become widely available for the assessment of functional abnormalities in the CF lung (**Figure 1**) (11, 12). Besides, a multitude of novel functional techniques based on inhalational contrast agents such as hyperpolarized gas MRI using alternative nuclei, or non-contrast-dependent techniques such as Fourier Decomposition MRI and T1 mapping have driven deeper insight into structure-function relationships in cystic fibrosis lung disease (13–18). Logically, evidence from CF research as a model has been transferred to rarer pediatric lung diseases and vice versa, such as primary ciliary dyskinesia or BPD (19, 20).

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Jim Wild, The University of Sheffield, United Kingdom

***Correspondence:**
Mark O. Wielpütz
mark.wielpuetz@med.uni-heidelberg.de

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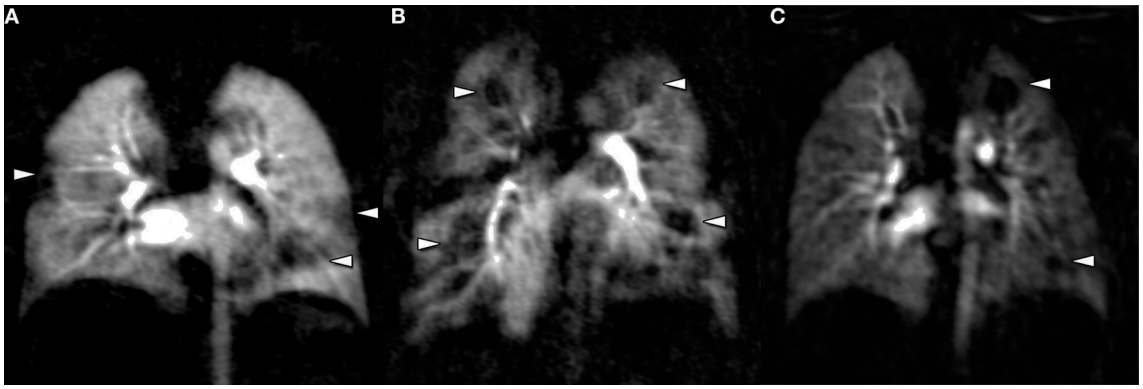

FIGURE 1 | Perfusion MRI is a ready-to-use technique to study functional impairment in pediatric lung disease, based on gadolinium enhancement. Perfusion abnormalities can be found in conjunction with structural lung disease in cystic fibrosis **(A)**, Langerhans cell histiocytosis **(B)**, or bronchopulmonary dysplasia **(C)**. Of note, perfusion abnormalities are non-specific and must be reviewed together with morphological imaging studies.

Although MRI has the advantage of relatively easy repeatability and "no dose," its application in neonates and infants, especially in prematurity, may be cumbersome due to sedation, acquisition time, MRI-compatible equipment, lines, and drug delivery, and clinicians might demand a quick examination with highest structural resolution. Specific MRI scanners for neonates will probably remain a rare research tool in the very near future (21). In this case, "low dose" may be acceptable, and CT can play to its recent advantages in speed and clarity of details (22). Most recent works focus on the use of "ultra low dose" CT with a radiation dose close to a conventional X-ray, and will very quickly redefine the aforementioned case-by-case balance between the benefits and detriments of an imaging study.

At this point, the authors Semple et al. (23) step in and elucidate on the recent advancements in radiation dose reduction of CT techniques, and developments in MRI and lung ultrasound to study BPD in infants and young children, and bring in their personal long-standing experience in CF lung imaging research. They provide a short introduction into the growing clinical relevance of BPD in pediatric medicine, and why imaging will play an important role for diagnosis and follow-up, similar to what has been discussed in CF. With regard to CT in BPD, the most relevant developments are summarized concisely, offering the technical specifications applicable in routine medicine. The frontiers of research on quantitative imaging and implications for standardization of CT imaging parameters similar to other diagnostic tests are only briefly touched upon.

MRI is presented as a research tool for BPD, and most studies cited and discussed in the present manuscript refer to studies performed in CF patients, whereas actual evidence for MRI in BPD is relatively low, mostly due to the limitations mentioned above in very young children and high technical effort to achieve structural detail in their very small lungs. BPD as a disease is even detrimental to the conditions for lung MRI, because of regional tissue and thus proton loss ("minus pathology") (24). Apart from the aforementioned neonate-specific prototype MRI (25), Förster et al. have added further BPD-specific evidence for the use of clinical state-of-the-art MRI for studying the pathophysiology of BPD in neonates. The authors found a decrease in T1 and an increase in T2 relaxation times were associated with an increased risk for BPD, which can partially be explained by tissue loss and perfusion changes in the diseased lung (26), and which was found also in COPD and CF (17, 27). Such findings may support the use of quantitative MRI for risk stratification and monitoring in BPD.

One should not forget about the potential of ultra-sound for pediatric lung imaging in the hands of experienced and ambitious examiners, which is also its main drawback—high levels of training required, and limited inter-observer agreement together with very limited overview render it an ancillary technique, and Semple et al. emphasize that in the context of BPD and intensive care treatment, X-ray remains the mainstay of everyday routine imaging to monitor lines and tubes, and also parenchymal lung changes.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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