

Chronic Lung Disease in the Preterm Infant: Lessons Learned From Animal Models

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ABSTRACT

Neonatal chronic lung disease (nCLD), also known as bronchopulmonary dysplasia (BPD), is the most common complication of premature birth, affecting up to 30% of very low birth weight infants. Improved medical care has allowed for the survival of the most premature infants, and significantly changed the pathology of BPD from a disease marked by severe lung injury, to the “new” form characterized by alveolar hypoplasia and impaired vascular development. However, increased patient survival has led to a paucity of pathologic specimens available from infants with BPD. This, combined with the lack of a system to model alveolarization *in vitro*, has resulted in a great need for animal models that recapitulate key features of the disease. To this end, a number of animal models have been created, by exposing the immature lung to injuries induced by hyperoxia, mechanical stretch, and inflammation, and most recently by the genetic modification of mice. These animal studies have: (i) allowed insight into the mechanisms that determine alveolar growth; (ii) delineated factors central to the pathogenesis of nCLD; and (iii) informed the development of new therapies. In this review, we will summarize the key findings and limitations of the most common animal models of BPD, and discuss how knowledge obtained from these studies has informed clinical care. Future studies should aim to provide a more complete understanding of the pathways that both preserve and repair alveolar growth during injury, which might be translated into novel strategies to treat lung diseases in both infants and adults.

Key words: neonatal chronic lung disease, bronchopulmonary dysplasia, BPD, animal models, preterm neonates, lung development, transgenic models

INTRODUCTION

Infants born prematurely frequently develop respiratory failure secondary to biochemical and structural immaturity of their lungs, and insufficient respiratory drive. While mechanical ventilation (MV) and oxygen rich gas are life-saving treatments, these therapies also promote lung injury. Thus, greater than 30% of preterm babies born prior to 30 weeks gestational age develop neonatal chronic lung disease (nCLD), also known as bronchopulmonary dysplasia (BPD), a disease with significant morbidity and mortality. Infants with BPD are at increased risk for long-term hospitalization and recurrent respiratory ailments in early infancy, as well as life-long consequences from impaired pulmonary and neurologic development (1-8). Affected infants may remain oxygen dependent for months, and although few remain oxygen dependant beyond two years of age (4, 9), respiratory symptoms, reflecting disturbed lung growth, are often evident for many years (5, 9-14). Extreme prematurity even in the absence of BPD appears to have long-term effects on lung function, with the majority of VLBW infants without a history of BPD demonstrating evidence of airway obstruction and diffusion defects years later (15). How BPD or prematurity itself alters pulmonary aging, and whether this results in early lung function decline, or increases the risk for adult lung diseases such as COPD is an ongoing discussion (8).

During the past two decades, advances in medical therapy have greatly improved the survival of premature infants. The use of antenatal steroids to accelerate lung maturation, the development of surfactant replacement therapy for acute respiratory failure, the institution of lung protective strategies of ventilation, and an optimization of nutritional support have all contributed to an overall decrease in the mortality of very low birth weight (VLBW) infants. Accompanying this increase in survival, the clinical, radiographic, and pathological features of BPD have changed significantly. The lung pathology of infants with the "old form" of BPD originally described by Northway et al., was characterized by evidence of severe lung injury including inflammation, protein-rich lung edema, extensive airway epithelial metaplasia, peribronchial fibrosis, and marked airway and pulmonary vascular smooth muscle hypertrophy (16-18). In contradistinction, birth of VLBW infants during the canalicular and saccular stages of lung development appears to disrupt the normal program of alveolar and vascular development, resulting in the "new BPD", characterized by alveolar simplification, dysmorphic capillaries, and increased in vascular and airway smooth muscle cells (19-22). Abnormal deposition of the extracellular matrix (ECM) components (e.g. elastin and collagen), and interstitial fluid accumulation are also observed (23-25).

Although of great clinical relevance, elucidating the pathophysiology of nCLD in the post-surfactant era has become increasingly challenging. With the reduction in BPD-associated mortality, the availability of pathologic specimens has decreased. Furthermore,

there is a paucity of *in vitro* systems that effectively model the complex 3-dimensional processes of alveolar formation and vascularization. Thus, defining the pathophysiology of BPD has relied, to a large extent, on the detailed observations made in animal models that recapitulate many features of this condition. Knowledge gained from these animal models has contributed great insight into the pathophysiology of both the 'old' and 'new' BPD, and led to changes in the clinical treatment regimen.

This review details some of the most extensively utilized animal models of BPD, and summarizes the information learned from these models including knowledge regarding the key molecular pathways, including growth factor signaling, that direct alveolarization, vascular development and ECM composition, and how inflammation, mechanical ventilation and oxygen treatment adversely affect these processes. This is followed by a discussion of how data obtained from animal studies have been translated into current treatment strategies for the care of premature infants.

OVERVIEW OF ANIMAL MODELS OF BPD

Numerous animal models have been developed to study the impairments in lung development that characterize BPD. Many of the commonly used models induce lung injury by: (i) altering the amount of inspired oxygen; (ii) applying stretch through mechanical ventilation; or (iii) inducing pre- or postnatal inflammation. Further, the recent development of genetically modified murine models, have allowed the identification of key molecular pathways that direct the individual components of alveolarization. Figure 1 schematically summarizes key findings from animal studies, relating the effects of hyperoxia and mechanical stretch to the characteristic inflammatory changes reproduced by different models of BPD. The resulting remodeling of the ECM and alteration in growth factor signaling alters epithelial, endothelial and (myo)fibroblast survival and proliferation, culminating in disruptions in pulmonary capillary development and distorted alveolarization.

Hyperoxia

The recognition that high levels of inspired oxygen are detrimental to the lung was made by investigators over one half century ago. Following the seminal publication by Northway et al, first describing the radiographic and histologic features of BPD, these same authors demonstrated that exposure of newborn guinea pigs to hyperoxia resulted in radiographic abnormalities that resembled the early stage of BPD in premature infants (26, 27). The injurious effects of oxygen can be replicated in many species; however, rodents are particularly well-suited to model BPD given that the newborn rodent is born during the saccular stage of lung development. Exposure of newborn mice to 100% oxygen for 7 days was shown to result in an initial phase of acute injury (including pulmonary edema and hemorrhage), followed by a chronic repair phase, characterized by fibroblast proliferation and collagen deposition (28). Further studies then demonstrated that hyperoxia not only induces lung injury, but also disrupts lung structure, affecting both alveolarization and vascularization. Exposure of newborn rats to 100% oxygen during the first week of life causes airspace enlargement that remains evident weeks later (29). Subsequent experiments demonstrated that even more moderate amounts of oxygen (60-85% FiO₂) cause durable impairments in lung structure (30), alter growth factor expression and decrease lung cell proliferation (31), schematically depicted in Figure 1.

These detrimental effects of hyperoxia on lung cells have been replicated *in vitro*. High levels of oxygen suppress alveolar epithelial type II proliferation via posttranscriptional mechanisms (32). Oxygen levels above 50% also impair endothelial proliferation, in part via the inactivation of fibroblast growth factors (33), reduce the numbers of endothelial progenitor

cells (EPC) in the blood, lung, and bone marrow in neonatal mice (34), and alter the function of side population of resident lung cells believed to have endothelial potential (35).

Many of the pulmonary responses to hyperoxia are developmentally regulated. Chronic oxygen exposure induces opposite apoptotic and proliferative responses in neonatal and adult lung cells in rats (36). In mice hyperoxia reduces EPC in the developing lung but not in adult animals (34), and in clinical studies, hyperoxia impairs the growth of endothelial colony-forming cells obtained from preterm but not from term infants (37). Furthermore, oxygen induced pulmonary vascular disease is age-dependant in mice, and neonatal exposure to hyperoxia appears to influence adult cardiopulmonary function and life-span in adults (38), and to durably alter pulmonary immune and oxidative stress responses (39, 40). Some of these sustained effects may be the results of epigenetic changes, related to the ability of oxygen to alter histone deacetylase activity in the neonatal mouse lung (41), with evidence suggesting that DNA methylation mediates neonatal programming of oxygen sensitivity in adulthood (42).

In summary, models exposing the immature lung to hyperoxia are clinically relevant. However, it must be recognized that the oxygen concentrations used in most of the above mentioned studies exceed the levels of oxygen supplementation currently applied to the preterm infant. In addition, animal models of hyperoxia lack the fluctuations in oxygen concentrations that are clinically observed in preterm infants, which could potentially induce differences in molecular signaling not reproduced in the experimental setting. Nonetheless, the ability to standardize these models, the good reproducibility, and the ability to perform studies addressing long-term follow up after neonatal hyperoxic injury, make the model attractive to many researchers in the field, and have allowed for its significant contributions to our current understanding in BPD.

Mechanical Stretch of the Premature Lung

The short-term mechanical ventilation of premature lambs and baboons were models initially developed to explore hyaline membrane disease (HMD) (43, 44). Subsequently the application of chronic mechanical ventilation resulted in the creation of novel animal models of BPD. An early study reported the effects of mechanically ventilating a small group of premature baboons with levels of oxygen ranging from 95-100%, for 8-17 days, all of which developed histopathologic evidence of BPD (45, 46). Further studies in this preterm baboon model reinforced the notion that hyperoxia is damaging to the immature lung, by demonstrating the universal appearance of BPD in premature baboons ventilated for up to 11 days with an FIO₂ of 1.0, in contrast to the absence of BPD in animals ventilated with oxygen

supplemented only on a 'prn' basis (47). This premature baboon model, originally developed in the pre-surfactant era, has been subsequently modified, including antenatal exposure to maternal glucocorticoids, postnatal surfactant treatment, and assisted ventilation with more modest inflating pressures and concentrations of inspired O₂, allowing it to better recapitulate the 'new BPD' (48). A similar strategy of mechanical ventilation with O₂-rich gas also induces lung pathology consistent with BPD in premature lambs (48-59). These studies in larger animals were integral in allowing mechanistic insight into the pathology of BPD, and permitting the testing of therapeutic strategies currently used in the care of premature infants, including surfactant replacement therapy (60), high-frequency oscillatory ventilation (HFOV) (61), and nitric oxide (54).

While the clinical condition and postnatal management of these premature animals closely resembled infants with BPD (48, 58), the costs and structural demands of these experiments using large animal models are substantial. Thus, additional models of BPD were developed by applying similar strategies to smaller animals. Mechanical ventilation of preterm rabbits was used to study HMD (62), and to evaluate therapeutic interventions such as surfactant replacement and inhaled glucocorticoids. Mechanical ventilation of newborn rats induces airspace enlargement and decreases alveolar numbers by 24h (63), and a similar effect occurs with the mechanical ventilation of newborn mice (64). This latter model, while technically challenging given the small size of the mouse pups, has the great benefit of allowing investigators to take advantage of genetically modified mice to more mechanistically explore key molecular targets (65-67). However, limitations of these murine models include the application of these injuries in pups born at term, and the inability to maintain these smaller animals for chronic, ventilation experiments.

Figure 1 summarizes the inflammatory changes and disruption of the ECM that result from mechanical stretch on the developing lung, leading to alterations in growth factor signaling that affect both endothelial and epithelial cells.

Infection, inflammation and their role in BPD

Evidence suggests that both pre- and postnatal inflammation contribute to the pathogenesis of BPD. Clinical studies demonstrate an imbalance in pro- and anti-inflammatory cytokines in tracheal aspirates from infants who later acquire BPD, which has been reviewed in detail (68, 69). The association between chorioamnionitis with the subsequent development of BPD (72) led further support to the notion that in some cases, BPD may either have a prenatal inflammatory origin, or may be aggravated by early inflammation (19, 73, 74). The cellular inflammatory response observed in experimental and

clinical BPD is dominated by the influx of neutrophils and macrophages into the lungs, and heightened neutrophil-derived elastase activity BPD (67-71). Neutrophils are activated in response to mechanical ventilation (71), and neutrophils are persistently elevated in the BAL fluid obtained from premature infants that develop BPD (72). LPS disrupts lung branching in early saccular mouse lung explants, an effect that depends upon NF κ B activation in macrophages (73). Additional inflammatory cells also appear to play a role in BPD. Connective tissue mast cells accumulate in the lungs of preterm infants with BPD(74), and data suggest that autoreactive T-cells may contribute to the premature baboon model of BPD (75).

Given that clinical studies cannot discriminate between the specific impacts of individual contributors to the pathogenesis of BPD, animal models using antenatal infection, endotoxin exposure, and/or transgenic mice have been developed to explore the link between lung inflammation and BPD. Antenatal endotoxin administration in rats (76) arrests alveolarization, and in preterm lambs, this effect appears to be time and dose-dependent (77). Studies in the premature baboons suggest that the efficacy of the immune response plays an important role in determining the ultimate outcome of perinatal inflammation of lung development. Animals that remain colonized with *Ureaplasma urealyticum* (Uu) require more O₂ and ventilatory support, and show more severe lung inflammation at necropsy (53). In contrast, lung function and O₂ need was less in preterm baboons that eradicate Uu. Even inflammation in the absence of infection leads to structural remodeling of the lung, including altered organization of ECM components, and impaired alveolarization and angiogenesis. The combination of hyperoxia and MV, a stimulus that impairs alveolarization, induces airway inflammation and cytokine expression in premature baboons (48), rodents, and rabbits (78-82). These inflammatory processes have been shown to be completely independent from the presence of pulmonary infections (83, 84). Moreover, the combination of these stimuli potentiates lung injury in sheep and mice (85, 86).

Additional animal studies have begun to elucidate the signaling molecules mediating these pathologic processes. Over-expression of IL-1 β in lung epithelial cells disrupts postnatal lung morphogenesis in the mouse (87, 88). In this model, MMP-9 plays a protective role, with MMP-9 deficiency further exaggerating the alveolar hypoplasia (89). In mice, cytokine production induced by ante- or perinatal endotoxin directly correlates with toll-like receptor 4 (TLR4) expression (90). Intra-amniotic endotoxin increases the levels of IFN-gamma-inducible protein-10 (IP-10) and monokine induced by interferon-gamma (MIG) in preterm lamb lungs, leukocyte chemoattractants that are angiostatic (91). In contrast, intra-amniotic exposure of mice in the early saccular stage of development stimulates angiogenesis, and increases the expression of angiogenic cytokines (MCP-1 and MIP-1 α in

fetal lung explants (92). Figure 1 underlines the critical role of inflammatory cells such as activated macrophages and neutrophils in the production of cytokines, proteases and other injurious factors that remodel the ECM and disrupt alveolar and vascular development.

DISRUPTION OF MOLECULAR PATHWAYS CONTROLLING ALVEOLARIZATION

The introduction of genetically modified mouse models has allowed the elucidation of numerous signaling pathways that are essential for lung development. As an example, the dependency of alveolarization on pulmonary vascular development was first revealed by studies examining vascular endothelial growth factor signaling in mice (93). A comprehensive discussion of the molecular pathways contributing to alveolarization can be found in a number of excellent recent reviews (94, 95). Here we will focus on how animal models have contributed to our understanding of key components of alveolar development and its pathology: (i) the development of the pulmonary capillary bed; (ii) remodeling of the ECM, and iii) the role of (altered) growth factor signaling. Here, the transforming growth factor (TGF) β /bone morphogenic protein (BMP) family serves as an excellent example given that it plays a central role in both vascular development and matrix remodeling, and is altered by hyperoxia, stretch and inflammation.

Pathways Directing Vascular Development

Pathologic examination of lung tissue from animal models and patients with BPD reveals fewer small arteries and an abnormal distribution of vessels within the distal lung (96-98). These and other data lead to the formation of the vascular hypothesis of BPD, wherein disruption of pulmonary angiogenesis induces the impaired alveolarization characteristic of BPD (19). In contrast, some studies have suggested that the pulmonary vascular network may be increased in BPD. Analysis of postmortem lungs from preterm and near-term infants exposed to short-term or long-term ventilation found that mechanical ventilation was associated with an expansion of the distal pulmonary circulation. However, this expanded network was abnormally formed, retaining the primitive dual capillary network characteristic of the saccular stage of development, with simplified non-branching vessels(99). Data from LPS-treated mouse lung explants suggest that inflammation may simulate abnormal angiogenesis in the developing lung via the up-regulation of angiogenic CC chemokines such as macrophage inflammatory protein-1 α (MIP-1 α) and monocyte chemoattractant protein-1 (MCP-1) (92).

Nonetheless, extensive experimental evidence derived from animal models has provided support for the notion that vascular development is an essential component of normal alveolarization, and that stimuli that block pulmonary angiogenesis disrupt secondary

separation. Administration of an antibody against platelet endothelial cell adhesion molecule - 1 (PECAM-1) in neonatal rats inhibits endothelial cell function and disrupts alveolarization (100). Chronic ventilation of preterm baboons prevents the physiologic increase in PECAM-1 protein and capillary density normally observed in utero, in keeping with human studies that demonstrate that the capillary network fails to expand in patients with BPD. Additional studies using a variety of models have begun to identify the specific signaling pathways that drive angiogenesis in the developing lung. Two of the predominant and best-studied pathways are discussed in detail below.

Hypoxia-Inducible Factor (HIF)

Members of the HIF family of transcription factors are tightly regulated by oxygen tension, and are key mediators of angiogenesis during development and disease. Under conditions of low oxygen tension, HIF is stabilized, translocates into the nucleus, and binds to hypoxia-responsive elements (HRE) located within the promoters of target genes, thus activating genes that increase oxygen delivery and allow for the metabolic adaptation to hypoxia. The transition of the pulmonary circulation from the hypoxic, fetal environment to the relatively hyperoxic postnatal state, likely results in rapid molecular changes mediated via oxygen sensitive pathways. HIF molecules play a key role in mediating the initial pulmonary adaptation and further lung maturation, in part by regulating the expression of the HIF downstream target, VEGF.

A role for the HIF pathway in regulating developmental angiogenesis was demonstrated by the targeted deletion of HIF family members in mice. Loss of *HIF1 α* in mice results in embryonic lethality at E10.5, with null embryos demonstrating multiple cardiac malformations and abnormally dilated blood vessels, perhaps due to the death of supporting mesenchymal cells (101). Targeted deletion of ARNT, the dimerization partner for both HIF-1 α and HIF-2 α , also results in embryonic lethality at E10.5, with affected embryos displaying defective angiogenesis of the yolk sac and branchial arteries (102). Interestingly, while embryonic lethality resulting from the absence of HIF-1 α appears to be independent of VEGF, loss of HIF-2 α in mice decreases alveolar epithelial cell expression of VEGF and causes fatal RDS due to insufficient surfactant production by alveolar type II cells (93), and a similar phenotype is induced by deleting the HRE located within the VEGF promoter.

In keeping with this data, preterm lambs suffering from RDS have decreased levels of both HIF-1 α and HIF-2 α , in association with diminished VEGF mRNA expression, and a corresponding increase in the expression of the HIF inhibitor, PHD-2 (103). In newborn mice, HIF-2 α gene expression increases during early alveolarization, but hyperoxia suppressed this increase (104). However, in that study, HIF-2 α levels did not correlate with VEGF levels

in the hyperoxia exposed group, suggesting that additional factors may regulate VEGF in hyperoxia, and suggest that some effects of disrupted HIF-2 α signaling may be independent of VEGF. Interestingly, over-expression of an oxygen-insensitive form of HIF-2 α in alveolar type II cells, also disrupts alveolarization, resulting in an abnormal accumulation of type II cells, decreased numbers of type I cells, and abnormalities in the pathways directing surfactant synthesis (105). These data suggest that HIF-2 α may play a role in alveolar maturation, and that tight spatial and temporal control of HIF-2 α is essential for normal lung development. Stabilizing active HIF with the pharmacologic PHD inhibitor, FG-4095, enhances angiogenesis of human lung microvascular endothelial cells *in vitro* (106). Further, augmenting HIF signaling *in vivo* by PHD inhibition ameliorates the physiologic consequences of BPD in preterm baboons, increasing alveolar surface area and improving oxygenation and lung compliance (107).

Vascular endothelial Growth Factor (VEGF)

VEGF, a key endothelial cell mitogen and down-stream target of HIF, is a regulator of angiogenesis and fetal lung maturation. Studies in embryonic mouse tissue demonstrated that VEGF expression is temporally and spatially restricted, and suggest that VEGF links airway and blood vessel formation by stimulating neovascularization at the leading edge of branching airways (108). Dysregulation of this temporal and spatial control of VEGF is detrimental to lung development. Over-expression of VEGF in alveolar epithelial cells causes embryonic mortality, increasing the growth of the pulmonary blood vessels but disrupting branching morphogenesis and alveolar type I cell differentiation (109). However, intrauterine administration of anti-VEGF-R2 antibodies later in embryonic development induces RDS, perhaps by blunting alveolar type II surfactant production (93). Administration of similar antibodies in the perinatal period disrupts early alveolar development in mice (110). Inhibiting constitutive activation of nuclear factor- κ B, a direct regulator of VEGFR-2 during alveolarization, impairs pulmonary angiogenesis and disrupts alveolarization in neonatal mice (111). Moreover, VEGF signaling appears to be important in maintaining lung structure even outside of lung development, as chronic treatment of adult rats with the VEGF receptor blocker, SU5416, induces alveolar septal cell apoptosis and causes airspace enlargement consistent with emphysema (112).

Decreased VEGF signaling may be one mechanism leading to the reduced pulmonary capillary volume and impaired alveolarization observed in very preterm baboons (51), and numerous studies have demonstrated an essential role for VEGF in murine embryonic vasculo- and alveologenesi. The expression of both VEGF and one of its receptors VEGF-R1, are decreased in the preterm baboon model of BPD. Mechanical

ventilation of 2- to 4-day-old mice with 40% O₂ reduces gene and protein expression of VEGF-A and VEGF-R2 in the lung, and results in lung structural abnormalities consistent with evolving BPD (113). These studies suggested that enhancement of VEGF may have therapeutic potential for BPD. In contrast to the effect observed during embryonic development, over-expression of VEGF in newborn rats increases survival, promotes lung angiogenesis and prevents hyperoxia-induced alveolar damage (114). Furthermore, VEGF preserves endothelium-dependent vasodilation in fetal sheep, potentially by up-regulating eNOS expression, data that may be particularly important given that a sub-group of infants with BPD develop pulmonary hypertension.

The Role of Extracellular Matrix in Secondary Septation

The process of secondary septation, whereby secondary crests outgrow into the surrounding mesenchyme and allow for the division of the alveolar ducts into alveolar sacs, as well as the formation of the capillary bed significantly depend on the formation of a scaffold by the ECM, comprised of collagen, glycoproteins and elastin (115, 116).

Elastin Fiber Assembly

The onset of septa formation is heralded by the deposition of elastin at specific sites in the walls of the developing saccules, a paradigm that was informed in large part from data obtained from animal models. The essential role for elastin in lung development was established by studies showing that deletion of the *Eln* gene in mice leads to neonatal death from cardiorespiratory failure, reducing terminal airway branching, and impairing pulmonary vasculogenesis (117, 118). The role of elastin in BPD was also outlined in the premature lamb model, where impaired alveolar and microvascular development is associated with an accumulation of excessive and disordered elastin (56). A similar abnormal accumulation of elastin is noted on lung pathology from premature infants who have died from BPD (21-23). Cyclic stretch of the lung during postnatal growth, induces tropoelastin gene expression, thus leading to increased elastin deposition (56, 119-122). The abnormal abundance and distribution of elastin is especially notable in blunted secondary crests, areas where focal deposits of distal elastin normally define loci of future alveoli. Ventilation of newborn mice disrupts elastin deposition, increases elastase activity, and reduces the expression of proteins critical for elastic fiber assembly (113). Moreover, in this model, the alterations in elastin breakdown and deposition are associated with increased lung cell apoptosis and defective septation, similar to that observed in infants suffering from BPD (65). Interestingly, these changes to the ECM appear to be primarily induced by cyclic stretch in the absence of

hyperoxia, as similar studies demonstrated that ventilating mice with air (21%) induces the same pathologic effects (64).

In contradistinction, animal models of BPD using severe and prolonged hyperoxia to induce lung injury showed varying results with respect to pulmonary elastin and collagen expression. Hyperoxia inhibits fetal rat lung fibroblast proliferation, type I collagen gene expression, and net collagen production *in vitro* (123). However, other studies in neonatal mice and rats showed that hyperoxia increases pulmonary type I collagen, tropoelastin, and connective tissue growth factor (CTGF), a fibroblast mitogen that promotes collagen deposition (124, 125).

Data from clinical studies supports the notion that increased degradation of elastin is associated with lung injury and the development of BPD. Pulmonary inflammation increases elastase activity in the tracheal aspirates of premature infants, which likely impairs elastin deposition and alveolar formation by enhancing elastin breakdown (72, 126, 127). This idea is reinforced by the observation that infants with BPD, and mice and lambs exposed to cyclic stretch with oxygen-rich gas, exhibit elevated levels of urine desmosine, a sensitive marker of elastin breakdown (56, 128-130).

Extracellular Matrix Remodeling

Remodeling of the ECM is primarily driven by serine proteases (e.g. neutrophil proteases and trypsin), matrix metalloproteinases (MMPs), and the papain family of proteases (cathepsin B, H, K, L and S). MMP-1, MMP-2, and MMP-9 are strongly expressed during alveolarization in both humans and mice (131, 132), and degradation of the ECM is necessary for angiogenesis. However, enhanced proteolytic activity appears to be detrimental to the developing lung. Increased lung levels of MMPs (133), cathepsins, and cathepsin activity in bronchoalveolar lavage (BAL) fluid are found in baboon models of BPD (134, 135). Moreover, the balance of matrix proteases and their inhibitors may have a broader effect on lung function outside of influencing ECM remodeling. For example, the serine protease inhibitor B1, expressed by neutrophils and macrophages, protects against the degradation of surfactant proteins by neutrophil elastase in baboon models of BPD (135). Further studies are necessary to clarify how proteolytic activity in the lung is tightly regulated during normal development, and the mechanisms that alter this regulation during BPD.

The Transforming Growth Factor- β /Bone Morphogenic Protein Superfamily in Normal and Abnormal Lung Development

The transforming growth factor beta (TGF- β) superfamily, including the bone morphogenic proteins, plays an important role in lung development by influencing the cellular composition of the lung via the regulation of endothelial and epithelial cell survival(136), as well as regulating ECM production and remodeling.

The expression and localization of TGF- β /BMP family members change significantly during lung development in both mice and humans (137, 138). TGF- β is activated in the vascular and airway smooth muscle, as well as the alveolar and airway epithelium throughout late lung development, and active TGF- β signaling is required for normal late lung development (94). Similarly, BMP signaling is also increased during the saccular and alveolar stages of lung development, suggesting a role for BMP in septal and vascular development (138). BMP family members stimulate the proliferation and migration of EC by increasing the expression of angiogenic factors such as VEGF-R2(139), and stimulate pulmonary EC angiogenesis by activating the Wingless (Wnt) signaling pathway(140).

Studies in murine models where key components of TGF- β /BMP signaling were disrupted have highlighted the importance of these pathways on alveolar development. The absence of latent TGF- β binding protein-3, a modulator of TGF- β secretion and activation, transiently decreases TGF- β activity in the lung at postnatal days 4-6, altering cell proliferation, inhibiting septation, and inducing emphysematous changes in the lung parenchyma (141). Epithelial cell-specific abrogation of Alk3-mediated BMP signaling in the prenatal lung disrupts distal airway formation in mice (142). The functions of TGF- β in the lung are cell specific, with disruption of TGF- β type II receptor in epithelial cells delaying postnatal lung alveolarization, while disruption in mesenchymal cells resulting in mildly abnormal lung branching and organ defects such as diaphragmatic hernia (143). Over-expression of TGF- β 1 in the newborn rat lung induces pathologic changes consistent with BPD (144), which are prevented by adenoviral-mediated gene transfer of decorin, a natural inhibitor of TGF- β activity (145, 146). In contrast, null mutation of Smad3, a key downstream effector of the TGF- β pathway, initially results in a subtle lung phenotype characterized by failure of normal organization of the EC. However, this early phenotype culminates in the development of pulmonary emphysema in association with excessive MMP activity (147, 148). Thus, the balance of TGF- β signaling appears to be critically important to alveolar development, with both excessive and insufficient TGF- β activity detrimental to distal lung growth.

TGF- β signaling is disrupted in animal models of BPD. Ventilation increases the expression of TGF- β in preterm lambs as early as one day after initiation of MV (149). The impaired alveolarization and decreased respiratory compliance observed in neonatal mice

exposed to hyperoxia is associated with potentiated TGF- β , but diminished BMP signaling, and a similar imbalance in TGF- β /BMP signaling is observed in murine lung epithelial cells and primary lung fibroblasts exposed to 85% oxygen *in vitro*. As a counter player to fibroblast growth factor- β and interleukin-1 β , factors that lower elastin mRNA in human lung fibroblasts, TGF- β increases elastin by both transcriptional and post-transcriptional mechanisms (150). Exposure of primary lung fibroblasts to 85% O₂ enhances the TGF- β -stimulated production of tropoelastin, as well as type I collagen- α , tissue inhibitor of metalloproteinase-1 (TIMP-1), and tenascin-C (138). Exposure of primary alveolar type II (ATII) cells to 85% O₂ increases the susceptibility to TGF- β -induced apoptosis, whereas primary pulmonary artery smooth muscle cells are unaffected (138). Combined with evidence showing that TGF- β impairs keratinocyte growth factor-stimulated proliferation of ATII cells, these data suggest that increases in TGF- β induced by hyperoxia may contribute to the alveolar hypoplasia characteristic of BPD by enhancing apoptosis and decreasing proliferation of ATII cells (151).

FROM BENCH TO BEDSIDE: THE UTILIZATION OF ANIMAL MODELS TO DISCOVER AND IMPLEMENT NEW TREATMENT STRATEGIES

Numerous therapeutic strategies currently in use or being investigated to reduce the incidence of BPD in premature infants have evolved, in large part, from data derived from animal studies. Important trials tested the feasibility and effectiveness of nasal continuous positive airway pressure (nCPAP) in premature baboons and sheep as a means of minimizing the need for MV, decreasing lung inflammation, and reducing or preventing chronic lung injury(55, 152). Surfactant replacement therapy was first demonstrated to be efficacious in animal models of RDS (153), improving alveolar expansion and reducing hyaline membrane disease in premature primates (154), and improving oxygenation and ventilation in premature lambs (155). These and similar studies provided the rationale for clinical trials of surfactant replacement therapy including reports of short-term increases in oxygenation in preterm infants following surfactant replacement (156, 157), and finally a randomized controlled trial demonstrating significantly reductions in mortality (158), and in the development of moderate to severe BPD (159, 160). Similarly, animal models of RDS were also integral in establishing the beneficial effects of antenatal glucocorticoids, including improvements in lung function, decreases in lung water content, increases in mean alveolar volumes, and maturation of the surfactant system (161-163). Conversely, experimental studies also highlighted the detrimental effects of postnatal glucocorticoid administration on cerebral development and myelination (164, 165), cardiac and renal function, and lifespan (166, 167) (168). Thus, corroborating results from clinical studies that demonstrated both short-term (e.g. hyperglycemia, hypertension, growth failure) and long-term (e.g. cerebral

palsy, developmental delay) adverse effects of postnatal steroids (169, 170). Data derived from animal studies also supported a key role for retinoids in lung development and repair after injury (171-178), in keeping with results from randomized, controlled trials that showed that retinol treatment in VLBW infants significantly decreases the incidence of BPD (179, 180).

The role of nitric oxide (NO) in the regulation of perinatal pulmonary vascular tone has been well established, and animal models helped to inform the notion that decreased NO synthesis contributes to the development of BPD (181, 182). Prolonged MV of premature lambs and baboons diminishes NOS activity and eNOS expression (181) (59) (52), and deficiency of eNOS exaggerates the adverse effects of hypoxia on alveolarization (183). Further, administration of iNO to mechanically ventilated preterm lambs and baboons improves lung function and increases alveolar counts (54, 184), and reduces endothelial cell apoptosis in neonatal rats after VEGF inhibition (185). However, while data from these experimental studies suggested that inhaled NO (iNO) may decrease the incidence of death and BPD in premature infants (184, 186), results from clinical trials failed to demonstrate a clear benefit of iNO on the development of BPD (187, 188).

Finally, animal models remain essential for the investigation and testing of novel strategies to prevent or treat BPD. Currently, there is great interest in studying whether cell-based therapies could be an effective treatment for BPD, including mesenchymal stem cells (MSCs), human amniotic fluid stem cells (hAFSCs) and endothelial progenitor cells (EPCs), comprehensively detailed in a recent review (189). Either intravenous or intratracheal administration of bone marrow-stromal cells (BMSCs) or the conditioned media (BMSC-CM) from these cells blunts the adverse effects of hyperoxia on the immature mouse lung (190, 191). In both studies, engraftment was low, and the beneficial effects of the BMSC-CM comparable or better than that of the BMSC, suggesting that the importance of a paracrine-mediated mechanism. Further experimental studies will be essential to delineate the appropriate dose and timing of administration, and to provide long-term safety and efficacy data prior to translating these exciting studies to the clinic.

CONCLUSION – WHAT HAVE WE LEARNED?

During the past quarter-century, advances in medical care of premature infants, including the widespread use of antenatal steroids and surfactant has greatly improved the survival of VLBW infants. However, with this increase in survival, the main focus of perinatal care is now on the prevention of long-term complications of prematurity. Neonatal CLD, the result of lung injury induced by oxygen, mechanical stretch and inflammation, superimposed upon functionally and structural immature lung, still accounts for one of the most important causes of impaired long-term outcome in the preterm infant. The increasing paucity of human tissue available from patients with BPD, and the absence of models that can recapitulate alveolarization *in vitro* have resulted in the development of numerous animal models that have been used to interrogate the pathogenesis of BPD. To date, information obtained using these models have permitted the identification of stimuli such as oxygen, stretch, and inflammation that are highly disruptive to the normal program of alveolar development. The development of genetically modified mice have permitted the elucidation of numerous molecular pathways that direct key components of secondary septation including the expansion of the pulmonary vascular bed via angiogenesis, and the remodeling of the ECM as well as how alterations in growth factor pathways, such as TGF- β /BMP, influence alveolar development. Moreover, these models have been integral in the development and/or implementation of therapies currently part of the standard of care for VLBW infants, including antenatal glucocorticoids, surfactant replacement, lung protective and non-invasive strategies of ventilation, and retinol therapy.

Data from animal studies have been integral in highlighting that secondary septation requires the complex interplay between multiple cell types within the lung, allowing for coordinated development of the pulmonary capillary bed and terminal airspaces, orchestrated in part by the deposition and remodeling of ECM. Distinct molecular pathways direct the individual components of secondary septation, and abrogation of a single pathway is often sufficient to disrupt the entire process of alveolarization. To this end, a number of injuries, including hyperoxia, mechanical stretch and inflammation impair alveolarization and contribute to BPD by altering one or more of these molecular pathways, resulting in impaired lung development by altering the differentiation, proliferation and migration of epithelial cells, endothelial cells, and myofibroblasts. Further, these injuries alter central growth factor signaling cascades that not only affect cell survival and fate, but also impact ECM composition.

Moving forward, the challenge will be to not only to understand the mechanisms regulating normal lung growth, but also to begin to clarify the pathways that mediate lung repair and regeneration after injury. Focus is needed on understanding how the separate

molecular pathways regulating alveolarization interact in a cell-specific manner at each stage of development, as well as during injury and repair, given that the global augmentation or inhibition of these signaling cascades (e.g. HIF and TGF- β /BMP) may negatively affect physiologic developmental and regenerative processes. Here, the identification of biomarkers that might herald the onset of BPD at early stages of the disease will be critical for the design of personalized treatment regimens. The mechanisms by which clinical variables, such as intrauterine growth retardation (192-194), and gender (195), influence the risk for BPD, need to be better addressed in animal studies. Similarly, a better understanding of the impact of the genetics on this heterogeneous disease, as suggested by twin (196), and genetic association studies (197, 198), is critical, and hold the promise of developing individualized treatments to more successfully prevent or treat BPD.

Further refinement and modification of the current animal models to better recapitulate the human condition would also be important in the future. This would include limiting the levels of oxygen used to cause lung injury in animals to those currently used in preterm infants, as well as models in which fluctuations of oxygen saturation are experimentally induced. In addition, a comparison of the structural abnormalities induced observed in animal models (i.e. sheep or baboons) at different stages of gestation treated with therapies more similar to current standard of care (steroids, surfactant, etc) might allow a better understanding whether the 'new' BPD is more the result of the greater immaturity of the infant at birth, or a reflection in the changes in medical care of premature infants that has occurred over the past 2 decades. Similar studies may also be helpful in understanding why late preterm infants (32-36 weeks gestational age) present with more long-term pulmonary and neurologic complications than anticipated. Finally, as the earliest survivors of BPD are now reaching adulthood, evidence is emerging that suggests that early injuries during alveolar development may represent the childhood antecedent of adult lung disease. Thus, the creation of "double-hit" animal models of disease could be integral in understanding whether mild injuries during lung development modulate physiologic aging of the lung, or alter the susceptibility to additional injuries later in life.

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Figure Legend

Figure.1: Pathophysiologic changes to the developing lung following exposure to oxygen and shear stress. Normal lung development is depicted on the left, abnormal processes on the right hand side.

