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Aberrant Signaling Pathways of the Lung Mesenchyme and Their Contributions to the Pathogenesis of Bronchopulmonary Dysplasia

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Abstract

Bronchopulmonary Dysplasia (BPD) is a chronic lung disease in infants born extremely preterm, typically before 28 weeks gestation, characterized by a prolonged need for supplemental oxygen or positive pressure ventilation beyond 36 weeks postmenstrual age. The limited number of autopsy samples available from infants with BPD in the post-surfactant era has revealed a reduced capacity for gas exchange resulting from simplification of the distal lung structure with fewer, larger alveoli due to a failure of normal lung alveolar septation and pulmonary microvascular development. While the mechanisms responsible for alveolar simplification in BPD have not been fully elucidated, mounting evidence suggests that aberrations in the cross-talk between growth factors of the lung mesenchyme and distal airspace epithelium play a key role. Animal models that recapitulate the human condition have expanded our knowledge of the pathology of BPD and have identified candidate matrix components and growth factors in the developing lung that are disrupted by conditions that predispose infants to BPD and interfere with normal vascular and alveolar morphogenesis. This review will focus on the deviations from normal lung development that define the pathophysiology of BPD and summarize the various candidate mesenchymal-associated proteins and growth factors that have been identified as being disrupted in animal models of BPD. Finally, future areas of research to identify novel targets affected in arrested lung development and recovery will be discussed.

Keywords

Lung development; septation; Bronchopulmonary Dysplasia; hyperoxia; signaling and growth factors

INTRODUCTION

Bronchopulmonary Dysplasia (BPD) is a chronic lung disease of prematurity defined by the prolonged need for supplemental oxygen (O₂) or mechanical ventilation beyond 36 weeks postmenstrual age (Jobe and Bancalari, 2001). Although advances in neonatal care have improved survival of extremely preterm infants, effective strategies to reduce the risk of BPD are lacking (Laughon, 2009) and 40% of extremely low birth weight (ELBW) infants continue to be affected annually (Fanaroff, 2007; Mathews, 2011; Stoll, 2010). Infants with BPD are more likely to require medications for pulmonary disease, be hospitalized in the first year of life, and suffer neurodevelopmental impairment (Anderson and Doyle, 2006;

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Ehrenkranz, 2005; Furman, 1996; Greenough, 2001; Schmidt, 2003). Moreover, the incidence of complications increases as the severity of BPD worsens (Ehrenkranz, 2005; Khemani, 2007).

BPD results from an interference with normal lung development triggered by the abnormal environment an infant is exposed to upon preterm birth (Baraldi and Filippone, 2007; Jobe and Ikegami, 2000; Jobe, 1999). The strongest risk factor for BPD is decreasing gestational age at birth (Laughon, 2011a; Rojas, 1995; Stoll, 2010), and infants at the extremes of viability (22–23 weeks) almost universally develop BPD (Stoll, 2010). Importantly, infants born at lower gestational ages are at a much earlier stage of lung development than their term counterparts and exposure to inflammation due to excessive O₂ supplementation, mechanical ventilation, chorioamnionitis with fetal involvement, or postnatal infection is hypothesized to interfere with the intricate pathways required for normal human lung development (Baraldi and Filippone, 2007; Jobe and Ikegami, 2000; Jobe, 1999; Morrissey and Hogan, 2009) and increase the risk of BPD (Ambalavanan, 2008; Bancalari, 2003; Finer, 2010; Groneck, 1994; Hartling, 2011; Jobe, 2005; Kraybill, 1989; Laughon, 2011a; Pierce and Bancalari, 1995; Watterberg, 1996). Recently, the association of BPD with intrauterine growth restriction (IUGR) (Bose, 2009; Laughon, 2011b; Zeitlin, 2010) and preeclampsia (Hansen, 2010) have suggested two additional risk factors for BPD possibly due to chronic hypoxia, aberrations in Vascular Endothelial Growth Factor (VEGF) signaling (Hansen, 2010) or long-lasting pulmonary vascular dysfunction (Jayet, 2010). Regardless of the antecedent, however, lungs of infants that die with BPD display abnormalities in the mesenchyme associated with an arrest of normal alveolar septation and pulmonary microvasculature development (Figure 1) correlating to the stage of lung development at birth, supporting the theory that BPD results from an inhibition of normal lung maturation (Albertine, 2010; Bhatt, 2001; Coalson, 2006; Coalson, 1999; Husain, 1998; Jobe and Ikegami, 2000; Lassus, 2001; Thibeault, 2003b).

The mesenchyme directs early lung development and likely influences late lung remodeling as well. Distal lung mesenchyme grafted onto the proximal airways can induce airway branching at ectopic sites (Alescio and Cassini, 1962; Taderera, 1967; Wessells, 1970) and cause differentiation of proximal epithelium into distal Type-II alveolar epithelial cells (Shannon, 1994; Shannon, 1998). The lung mesenchyme is complex and primarily composed of fibrous structural proteins (mainly Type-I and IV collagen, elastin, fibronectin, and laminins) secreted from fibroblasts that provide a supportive scaffold for the airspaces and airways. While collagen and elastin fibrils convey the majority of the physiologic properties to the lung parenchyma, fibronectins and laminins serve a vital function by linking cells to the larger fibrillar proteins of the matrix (Alberts, 2002). The structural proteins are woven within a supportive network of glycosaminoglycans and proteoglycans which regulate the water content of the mesenchyme and contribute to the physical consistency of the tissue. In addition, the glycosaminoglycans, including hyaluronic acid and heparan sulfate, can bind and concentrate growth factors in the appropriate locations, enhancing presentation to their receptors and influencing the overlying cells' behavior (Thompson, 2010). Additional mesenchymal proteins exist that do not directly contribute to structural or mechanical support but rather exert influence on the overlying cells, often via cellular integrins, and can modulate adhesion, migration, and cell survival. These “matricellular proteins,” aptly named for their function to link cells to the extracellular matrix, include the tenascins and thrombospondins (Bornstein and Sage, 2002; Schroeder, 2003). Collectively, these features of the mesenchyme suggest that it is an important component that, when disrupted, could potentially disrupt normal lung morphogenesis. Indeed, factors that predispose to BPD, including mechanical ventilation, infection, and hyperoxia, can all interfere with mesenchymal structure and function and have been associated with inhibition of normal lung development. Thus, an understanding of the

contributions of the mesenchyme to lung development may potentially help identify and treat deleterious adaptations that cause BPD and reduce its burden in preterm infants. This review will focus on animal models of arrested alveolar development that have shaped our current understanding of the pathophysiology of BPD, as well as discuss some of the aspects of alveolar development that remain poorly understood and serve as areas of future research to identify novel lung development targets affected in BPD.

Normal Human Lung Development

An understanding of the normal stages of lung development and their relation to the timing of preterm birth is key to identifying which processes are impacted in BPD. The lung first appears at 3 weeks gestation as a bud from the primitive foregut and, through a series of dichotomous branching during the embryonic and pseudoglandular stages directed by signaling between the mesenchyme and epithelium, the conducting airways of the lung are fully formed by 16–18 weeks gestation (Rutter and Post, 2008; Wert, 2004). Additionally, the terminal bronchioles have branched into two or more respiratory bronchioles, each giving rise to clusters of acinar tubules and buds which will form the future alveolar ducts and alveoli, respectively. It is presently unknown if this early stage of lung development is disrupted by inhibitory factors like chorioamnionitis, as it is likely that these pregnancies would deliver at pre-viable gestations. Preterm infants with BPD that were born to pregnancies complicated by chorioamnionitis may have been exposed at these early stages of development, but it is difficult to determine exactly when intrauterine infection first appears and is clinically-relevant.

The canalicular stage occurs between 18–26 weeks gestation (embryonic day “E”16.5 to E17.5 in mice) and is most likely the earliest period of lung development impacted by preterm birth. Fitting with the pathology of BPD, it is the period of extensive distal airspace structural and microvascular development. Throughout the canalicular stage, the acinar tubules and buds lengthen, subdivide, and widen while the surrounding mesenchyme progressively thins. This stage is characterized by increasing proliferation of distal lung epithelial cells and rapid expansion of the intra-acinar capillaries. The primitive, cuboidal distal lung epithelial cells differentiate into (1) immature Type-II alveolar cells that begin producing phospholipids and surfactant proteins and (2) squamous Type-I cells that appose the expanding capillaries to form the primitive respiratory epithelium capable of gas exchange. Viability of the preterm infant is limited by the appearance of this functional gas-exchange unit, and antenatal steroids and exogenous surfactant replacement have increased survival at this stage of lung immaturity by promoting endogenous surfactant production as well as accelerating the thinning of the mesenchyme required to allow diffusion of gas into the blood (Ballard, 1989; Fanaroff, 2003; Roberts and Dalziel, 2006; Whitsett, 1987). Thus, infants now surviving birth at the extremes of gestation are subjected to an environment that is much different from the intrauterine and can potentially disrupt the normal processes (including orchestrated changes in the mesenchyme) required for alveolar airspace formation.

The saccular stage of lung development occurs between 24–38 weeks gestation (E17.5 to postnatal day “P”4) and is primarily responsible for the expansion of the pulmonary acinus. It is significant to note that infants born prior to 28 weeks gestation are at highest risk of developing BPD (Stoll, 2010), suggesting that disruptions occurring in saccular morphogenesis are primarily responsible for BPD pathogenesis. During the saccular stage, additional branching, lengthening, and widening of the acinar tubules and buds forms thin-walled alveolar saccules and ducts (Wert, 2004). Thinning of the surrounding mesenchyme and further differentiation of cuboidal acinar epithelial cells into squamous Type-I cells decreases the distance required for O₂ diffusion and expands the functional gas-exchange

capacity of the lung. By the end of the saccular stage, the relative contribution of the airspace to the total lung volume has increased dramatically at the expense of the surrounding mesenchyme (Rutter and Post, 2008). Importantly, it is during this stage that primary septa are primed for subdivision by secondary alveolar septa in the alveolar stage (Burri, 2006; Galambos and deMello, 2008; Morrissey and Hogan, 2009; Thurlbeck, 1975).

While the saccular lung has the structural components required for gas-exchange, the alveolar stage exponentially increases the surface area of the respiratory epithelium from birth through early childhood. Alveoli first appear in the human saccular lung at approximately 28 weeks and slowly expand to approximately 20% of adult numbers by 40 weeks (Hislop, 1986; Langston, 1984). The bulk of alveolarization occurs between birth and 6 months of age (P4 to P15 in mice) and continues throughout the first 2–3 years of life (P14 to P36 in mice), though it may also continue into adulthood (Burri, 2006; Schittny, 2008). While preterm birth occurs weeks before the expected initiation of secondary alveolar septation, alterations in the developing saccules appear to persist and are capable of inhibiting future alveolarization.

Orchestrated changes in the mesenchyme are necessary for formation of the secondary alveolar septa, highlighting its importance in normal alveolar development. Secondary septa originate from the relatively thick saccular wall and contain a double capillary network with a central core of delicate connective tissue (Figure 1A). Elastic fibers are situated within the primary septa and are at the initiation site of the secondary crests that grow and project into the airspaces (Burri, 2006; Roth-Kleiner and Post, 2005; Schittny, 2008; Thurlbeck, 1975). Myofibroblasts expressing alpha smooth muscle actin (α SMA) are located within the secondary crest and are proposed to secrete matrix proteins that elongate the secondary septa to subdivide the saccular airspace into a mature alveolus (Brody and Kaplan, 1983; Galambos and deMello, 2008; Morrissey and Hogan, 2009). (Figure 1B) The importance of proper myofibroblast differentiation and spatial distribution is suggested by multiple animal models of BPD that have demonstrated increased numbers and abnormal location of these cells in saccular walls that fail to septate. Finally, the pulmonary microvasculature matures from a double to a single capillary network, minimizing the distance required for gas diffusion. Interference with any of the above components of mesenchymal maturation results in failure of secondary septation.

Pathological Changes in Lung Mesenchyme and Development Associated with BPD

A limited number of autopsy samples from infants with gestational ages <30 weeks in the pre- and post-surfactant era that have died with BPD have been described, and abnormalities in the mesenchyme were consistently seen in association with inhibited alveolarization. Even prior to the introduction of prenatal steroids and surfactant, improvements in respiratory management and care of preterm infants had allowed very preterm infants <30 weeks and <1000g to survive, and the pathological features of BPD began to change (Hislop, 1987; Van Lierde, 1991). Rather than the destructive, emphysematous changes seen in the patients described by Northway (1967), infants in the era of gentle ventilation and conservative O₂ supplementation demonstrated diffuse alveolar simplification due to a lack of secondary septation. They continued to display abnormalities in the mesenchyme, manifested by interstitial fibrosis and thickening. However, mesenchymal abnormalities were more diffuse and homogenous than in the past and are now clearly associated with the arrested alveolarization.

Preterm infants born during the surfactant era have continued to display abnormalities in the mesenchyme in association with inhibited alveolarization. Hussain (1998) examined 14

surfactant-treated (gestational age 24–32 weeks) and 8 non-surfactant-treated (gestational age 27–29 weeks) infants with BPD in comparison to age-matched controls. Both non-surfactant-treated and surfactant-treated infants had alveolar simplification in association with interstitial fibrosis. Thiebault (2000) reported on 44 infants born at <30 weeks gestational age considered at risk for BPD that died within 2 months of birth. A significant proportion of the infants had received prenatal steroid therapy as well as surfactant. With increasing respiratory support in infants at risk for BPD, total lung elastic tissue and septal thickness abnormally increased in association with decreased internal surface area and alveolar duct diameter compared to gestational age-matched controls. In a corresponding study (Thiebault, 2003a), infants at greatest risk for BPD and need for respiratory support demonstrated increased total collagen content in the lung as well. In contrast to the delicate interstitial collagen fiber network normally seen in the developing lung, infants with BPD had thickened, tortuous and disorganized collagen fibers. Thus, it is thought that abnormal matrix development may have interfered with normal alveolar development within these infants.

While human lung samples have helped characterize the gross phenotypic changes of BPD in the present era, the insight they provide into the underlying mechanisms of abnormal lung alveolar development is unfortunately limited. Advances in medical care have improved the survival rate of infants with BPD and, fortunately, relatively few will die during the acute development of the disease. The result, however, is a limited number of autopsy samples available to study the early pathological changes associated with BPD. Secondly, autopsy specimens would likely represent end-stage lung disease with active secondary repair mechanisms that may be mistaken for primary inhibitors of alveolarization. Confounding factors that have been implicated in the pathogenesis of BPD such as inflammatory responses to infection, mechanical ventilation and supplemental O₂, may indeed be involved in the pathology of abnormal alveolar development but alternatively may also be reactions to unrelated insults. Thus, there has been a heavy reliance on animal models that recapitulate the phenotypic characteristics of BPD to gain a better understanding of not only of the pathophysiology of BPD but also the normal processes involved in alveolar development.

Large animal models that recapitulate the circumstances of extreme preterm birth have attempted to define the progression of alterations in lung development that culminate in BPD. The 125 day gestation baboon model, equivalent to a 24–26 weeks infant's lung developmental stage, has provided great insight into the impact of prematurity and neonatal care on lung development during the saccular stage (Coalson, 1999). After administration of antenatal steroids, preterm delivery occurred by C-section and animals were managed according to contemporary standard care practices, including early exogenous surfactant, gentle ventilation and judicious use of O₂. They developed radiographic and clinical features consistent with human BPD and, compared to term controls, had enlargement of saccular airspaces due to a lack of secondary alveolar septation (Figure 1C). The interstitium was of variable thickness and hypercellularity with increased mesenchymal cells and focal deposits of elastin and collagen. In addition, similar to findings in human preterm infants (Bhatt, 2001; Lassus, 2001; Thiebault, 2003b), the baboon pulmonary microvasculature was reduced, dysmorphic, and abnormally positioned in the thickened saccular walls. Interestingly, when this same model was utilized to investigate the effect of early nasal continuous positive airway pressure (CPAP) on lung development (Thomson, 2004), CPAP-treated animals lacked the previously-identified pathological changes of the interstitium associated with mechanical ventilation and had normal distal airspace septation. These findings reinforce the notion that distal airspace development is dependent on a properly specified and organized mesenchyme.

Alterations in Mesenchymal-Associated Proteins and Growth Factors Associated with Animal Models of Alveolar Septation

Perhaps the most convincing evidence that the mesenchyme plays a central role in the pathophysiology of BPD comes from models of arrested alveolar development where many alterations in matrix-associated proteins and growth factors are seen. Because large animals do not have the availability of transgenic strains, genomes cannot be manipulated and aberrant pathways may be mistaken for primary effectors when they are merely secondary responses to inflammation unrelated to the mechanisms of alveolarization. Rodent models have, therefore, been developed that closely mimic human BPD and have several advantages over large animals. Rats and mice are born with saccular lungs similar to preterm infants, and transgenic technology allows investigation of the contribution of multiple genes required for lung developmental (Kimura and Deutsch, 2007). Because secondary alveolar septation occurs shortly after term birth in mice (mainly from postnatal day 4 to 14) (Schittny, 2008), they offer an attractive model to study particularly the aberrations in saccular and alveolar lung development. The most extensively used model to study arrested alveolar development in rodents has been the hyperoxia model. Exposure to 60–85% O₂ from birth through the saccular and alveolar stages efficiently disrupts secondary alveolar septation, resulting in a simplified distal airspace due to developmental arrest at the saccular stage (Alejandro-Alcázar, 2007; Auten, 2009; Balasubramaniam, 2007; Dager, 2003; Velten, 2010; Warner, 1998; Yee, 2006). Observations from this model have identified several candidate genes and proteins that are dysregulated in BPD and have led to studies utilizing genetic manipulation to determine if they play a vital role in alveolar lung development. The following is a summary of these data, with a focus on mesenchymal proteins and growth factors, obtained from models of BPD that have shaped our current understanding of the genetic mechanisms involved in aberrant distal lung development (Table 1).

Elastin

The vital role of the mesenchymal structural protein, elastin, in alveolar septation has been extensively studied in human and animal models of lung development. During normal lung development, tropoelastin gene expression first appears in the pseudoglandular stage and peaks at the time of alveolarization where it is localized to growing secondary septal tips in the saccular airspace (Mariani, 1997; Willet, 1999). In a sheep model of lung development (Willet, 1999), significant increases in elastin content in elongating secondary septal tips coincided with formation and maturation of secondary septa, and the same is true in studies of human lung development (Nakamura, 1990). Co-localization studies have identified the α SMA-positive myofibroblast as the major contributor of elastin to the growing secondary septal tips in both animals (Dickie, 2008; Noguchi, 1989; Vaccaro and Brody, 1978) and humans (Leslie, 1990).

The absolute need for elastin in alveolarization has been demonstrated in transgenic animal and observational human studies. Genetic ablation of elastin in mice prohibited the postnatal formation of secondary alveolar septa, despite normal saccular structures being in place (Shifren, 2007; Wendel, 2000). Likewise, disruption of appropriate elastic fiber assembly due to mis-expression of lysyl oxidase (Das, 1980; Kida and Thurlbeck, 1980; Kumarasamy, 2009; Liu, 2004) or deletion of fibrillins (Neptune, 2003) inhibited the normal postnatal expansion of the elastic fiber network and significantly impaired alveolarization, emphasizing the importance of proper elastin assembly in postnatal lung remodeling. Loss of platelet-derived growth factor alpha (PDGFA) in the developing lung resulted in failure of α SMA-myofibroblast migration into the saccular airways associated with a complete lack of elastin in the growing septa, confirming that these specialized fibroblasts are responsible

for elastin deposition in the developing lung (Boström, 2002; Boström, 1996; Lindahl, 1997). Recently, newborn mice injected with a PDGF receptor inhibitor from postnatal (P) day 1 to 7 demonstrated disorganized elastin deposition and complete failure of secondary septation resulting in enlarged, simplified alveoli that persisted into adulthood (Lau, 2011). Thus, elastin is required for normal alveolar septation, and PDGF signaling plays a key role in elastin deposition in the developing lung.

Disorganized elastin deposition has also been noted in the saccular walls of humans and animals that develop BPD (Thibeault, 2003a). Exposure to hyperoxia (Bruce, 1993; Bruce, 1996) or mechanical ventilation (Albertine, 1999; Nakamura, 2000) up-regulates elastin gene expression in association with impaired alveolar septation. However, normal elastin assembly in these animals was disrupted and the distribution of elastin was throughout the septal walls rather than the typical site of secondary septal formation. Thus, it is clear that organization of elastin in the growing septal tips is vital for alveolarization to proceed normally (Bland, 2008; Bland, 2007; Mokres, 2010), but the mechanism by which it drives alveolar septal formation remains understudied and poorly understood.

Fibroblast Growth Factors (FGFs)

FGFs are expressed in the mesenchyme of the developing lung during early branching morphogenesis, are required for normal development of the conducting airways (Peters, 1994) and, although their role in the saccular and alveolar stages has yet to be clearly defined, mounting evidence suggests that they are necessary for proper acinar development. In addition, FGFs are important regulators of lung cell migration, proliferation, and differentiation (Cardoso, 1997), important to all processes of lung development. While all four receptors (FGFR-1,-2,-3,-4) are expressed in the late embryonic/early postnatal rat lung, FGFR-2,-3,-4 mRNAs are maximally expressed in the alveolar epithelium during postnatal alveolarization (Powell, 1998). Disruption of FGFR-2 in the airway epithelium at the time of lung bud formation results in complete failure of lung branching due to its requirement for early branching morphogenesis (Peters, 1994), and spatiotemporally-controlled transgenic mouse expression of soluble dominant-negative(DN) FGFR-2 during the canalicular stage results in permanent emphysematous changes in adulthood (Hokuto, 2003). However, expression during postnatal saccular and alveolar development did not affect alveolarization, suggesting that abnormalities in late branching morphogenesis interfered with the proceeding secondary septal formation. A possible mechanism was suggested in a regenerative model of lung alveolarization, where expression of DN-FGFR-2 interfered with re-alveolarization and blocked the regenerative effects of retinoic acid by inhibiting the differentiation of elastin-producing α SMA-myofibroblasts (Perl and Gale, 2009).

While FGFR-2 appears to play a role in the late-canalicular/early-saccular stage of lung development, FGFR-3 and -4 likely contribute to postnatal alveolar lung development. Compound, systemic genetic ablation of both FGFR-3 and -4 results in a normal saccular lung, but there is failure of secondary septation with increased elastin gene expression and abnormal elastin deposition thereafter (Srisuma, 2010; Weinstein, 1998). Both FGFR-3 and -4 are decreased during the initiation of alveolar development in hyperoxic animal models (Park, 2007), but their expression in relation to BPD is unknown.

The FGF ligands, FGF-7 and FGF-10, are necessary for normal alveolar development and are reduced in BPD. FGF-7, also known as Keratinocyte Growth Factor, binds to FGFR-2 and stimulates proliferation of Type-II alveolar epithelial cells (Cardoso, 1997). In the immediate postnatal lung, it localizes to the airway and alveolar epithelium as well as the mesenchyme surrounding the pulmonary microvasculature (Padela, 2008). A direct role for FGF-7 in normal postnatal lung development has not yet been established, but its expression is eventually suppressed by hyperoxia (Park, 2007) and BPD risk is inversely proportional to

its level in tracheal aspirate in the first 5 days of life (Danan, 2002). Exogenous supplementation (Barazzzone, 1999; Franco-Montoya, 2009; Frank, 2003) or transgenic over-expression (Ray, 2003) can protect rodents from hyperoxia-induced cell death and alveolar epithelial damage, likely via activation of the AKT survival pathway, but it cannot reverse the inhibitory effects of hyperoxia on alveolar septation. However, when neonatal rats were given FGF-7-neutralizing antibodies immediately prior to alveolar septation, proliferation, peripheral microvascular development and secondary septal formation were adversely impacted and lungs displayed a characteristic BPD-like phenotype at P7 (Padela, 2008). Thus, although it is clear that FGF-7 protects alveolar epithelium by promoting cell survival and some expression is required for normal lung development, it is unable to reverse the pathological airspace simplification in BPD.

FGF-10 is vital for branching morphogenesis of the conducting airways (Sekine, 1999), and emerging evidence supports an equally-critical role in saccular lung development. Throughout branching morphogenesis and into the saccular stage of lung development, FGF-10 is expressed in the mesenchyme adjacent to the alveolar epithelium. Human infants with BPD demonstrate reduced expression of FGF-10 in association with inhibited saccular airway branching (Benjamin, 2007). Exposure of canalicular-stage lung explants to lipopolysaccharide (LPS) to mimic the effects of chorioamnionitis arrests saccular airway branching by reducing FGF-10 expression, and supplementation of FGF-10 can reverse the effect (Benjamin, 2007). In addition, α SMA-myofibroblasts in LPS-exposed explants were abnormally situated, forming thick bands within saccular walls rather than being positioned at growing septal tips. These effects appear to be due to direct activation of NF- κ B by LPS, which in turn interferes with factors that enhance FGF-10 promoter activity (Benjamin, 2010). Because infants at highest risk for BPD are born during the transition from the late-canalicular to early-saccular stage of lung development, therapies to maintain FGF-10 expression may promote saccular and, therefore, alveolar development.

Epidermal Growth Factors (EGFs)

Epidermal growth factors are present during normal lung development and dysregulation of their expression can adversely impact septation. The EGF ligands include Transforming Growth Factor alpha (TGF α), Amphiregulin, EGF, and heparin-bound EGF, all of which bind to the common EGF-receptor (Ruocco, 1996; Schlessinger, 2004). Both receptor and ligands are immunolocalized to mesenchymal cells surrounding the conducting and distal airspaces (Ruocco, 1996; Schuger, 1993) as well as the saccular epithelium (Strandjord, 1994) during lung development where they modulate fibroblast and epithelial cell proliferation (Derynck, 1986; Sundell, 1980) and promote terminal airway branching (Schuger, 1993). Mice devoid of EGF-R have defective lung branching as early as E12 and, therefore, display abnormal alveolar development as well as Type-II alveolar cell immaturity (Miettinen, 1995; Miettinen, 1997).

Although most EGF-R signaling appears to be beneficial to lung maturity, excessive TGF α is thought to be detrimental to distal airspace development. Infants that died of BPD had elevated TGF α in their lungs (Stahlman, 1989; Strandjord, 1995), and excessive exposure to TGF α interferes with normal terminal airspace development in animal models (Hardie, 1997; Hardie, 1996; Kramer, 2007; Le Cras, 2004; Le Cras, 2003). TGF α over-expression under the control of the surfactant protein C (SP-C) promoter in either the late canalicular/early saccular period (Kramer, 2007) or during the initiation of alveolarization (Le Cras, 2004) disrupted lung morphogenesis resulting in alveolar simplification and pulmonary hypertension. Impressively, transient SP-C-mediated over-expression from P3 to P5 was sufficient to dramatically interfere with secondary septation and disrupt elastogenesis resulting in fragmented, irregularly-distributed elastin fibers in the alveolar wall and inhibition of pulmonary microvascular development. These effects persisted into adulthood,

reinforcing the concept that a critical window exists when aberrations in the normal milieu of the neonatal lung can permanently disrupt alveolar development.

Transforming Growth Factor β (TGF β)

The Transforming Growth Factor Beta (TGF β) superfamily of growth factors, consisting of the TGF β s and Bone Morphogenic Proteins (BMPs), modulate cell survival (Heldin, 2009; Zhang, 2004; Zhang and Phan, 1999), differentiation (Ambalavanan, 2008; Valcourt, 2005), and ECM deposition (Blom, 2002; Grotendorst, 1996). TGF β consists of three ligands, TGF β -1, -2, and -3, that localize to the subepithelial region of the developing bronchioles in the late-canalicular/early-saccular lung (Pelton, 1991; Schmid, 1991). While all three ligands are present in the alveolar regions during the immediate post-natal period (Nakanishi, 2007), there is a lack of information about the cellular source of TGF β ligands or the relative proportion that each ligand contributes to total TGF β levels in the saccular lung. Furthermore, evidence from individual ligand deletion studies suggests that each serves overlapping, yet specific, functions (Kaartinen, 1995; Sanford, 1997). For instance, loss of TGF β -3 ligand results in immediate postnatal lethality due to failure of late embryonic distal lung development (Kaartinen, 1995), loss of TGF β -2 ligand results in collapse of the distal airways and immediate postnatal death from cyanotic cardiac malformations (Sanford, 1997), whereas loss of TGF β -1 does not affect lung morphogenesis and alveolarization (Shull, 1992). Thus, environments that create an imbalance in the proportions of ligand expression may impact lung development. Although BMPs are modulated by hyperoxia in models of BPD (Alejandre-Alcázar, 2007), they do not appear to play a primary role in alveolarization and BPD pathophysiology.

Recent studies connecting aberrations in TGF β signaling and postnatal lung maldevelopment have yielded mixed results, and it is unclear if TGF β inhibits or promotes alveolar septation. Preterm infants with increased levels of TGF β in tracheal aspirates at 4 days of age were more likely to progress to BPD than similarly-aged infants with or without respiratory distress syndrome (Kotecha, 1996), and elevated levels in the first 30 days predicted the need for O₂ at discharge. Neonatal mice exposed to hyperoxia have increased levels of TGF β (Alejandre-Alcázar, 2007; Nakanishi, 2007) concurrent with decreased BMP signaling (Alejandre-Alcázar, 2007), but only after 14 day of exposure when significant reparative fibrotic mechanisms are likely active and inhibition of alveolarization is already obvious. Over-expression of TGF β in the immediate postnatal period either by inducible transgene (Vicencio, 2004) or intranasal adenoviral vector (Gauldie, 2003) inhibited alveolarization but with a more pronounced degree of fibrosis than that seen in hyperoxia models. The strongest support for an inhibitory effect of TGF β on distal lung development comes from the finding that administration of a TGF β -neutralizing antibody during the period of hyperoxic exposure attenuates hyperoxia-induced hypoalveolarization (Nakanishi, 2007). However, transgenic silencing of TGF β signaling in postnatal hyperoxia exposure has not been reported.

Up-regulation of TGF β by hyperoxia has also been associated with inhibition of the PPAR-gamma pathway and may explain a possible mechanism by which TGF β could inhibit alveolar lung development. Neonatal rats exposed to hyperoxia for the first 24hr of life demonstrated an up-regulation of the TGF β pathway and impaired saccular airspace development (Rehan, 2010; Rehan, 2006), but pre-treatment with the PPAR-gamma agonist, Rosiglitazone, at E18 and 19 or during hyperoxia exposure preserved saccular airspace development associated with decreased TGF β signaling. Likewise, when neonatal rats were given Rosiglitazone while being exposed to 7 days of continuous hyperoxia, increases in TGF β signaling were abolished and alveolarization was preserved (Dasgupta, 2009), providing evidence to suggest that increased TGF β signaling actively inhibits alveolar septation.

Although increased TGF β signaling has been associated with inhibited postnatal lung development, equally-convincing evidence suggests that there is a minimum level of TGF β signaling required for normal alveolar development. Deletion of the Type-II TGF β receptor (TBR2, the common receptor for all TGF β ligand signaling) in the alveolar epithelium via the SP-C promoter effectively silenced TGF β signaling in the developing lung. Despite having normal-appearing lungs at birth, by P7 mice deficient in TGF β signaling had enlarged, simplified airspaces due to a lack of secondary alveolar septation associated with decreased proliferation of alveolar epithelial cells and reduced numbers of Type-I alveolar epithelial cells (Chen, 2008). Significantly, similar to BPD patients, these abnormalities persisted to at least 2 months of age (the endpoint of the study). Similarly, genetic ablation of Smad3, one of the two intracellular intermediates of TGF β signaling, resulted in distal airway over-simplification as early as P7 due to inhibited secondary alveolar septation despite having normal lungs at birth (Chen, 2005). Defects in distal lung cell proliferation were noted, similar to TBR2-KO mice, and septation failed to increase in adulthood. As the lungs became progressively more emphysematous with time, elevations of matrix metalloproteinase-9 (MMP-9) increased, suggesting TGF β signaling may play a vital role in maintaining homeostasis of the mesenchyme during lung alveolar development.

Connective Tissue Growth Factor (CTGF) is a downstream product of TGF β signaling that modulates fibrosis and has been linked to abnormalities in alveolar septation. During the pseudoglandular stage of development, CTGF is expressed in the epithelium of the terminal airways and, as gestation progresses, expression intensifies and extends into the surrounding mesenchyme (Burgos, 2010). Just prior to birth, however, expression in the distal airspaces diminishes significantly, although some expression remains in the mesenchyme. While TGF β is one well-known regulator of CTGF expression (Blom, 2002; Grotendorst, 1996), it can also be induced by hyperoxia (Alapati, 2011; Chen, 2007) and high-tidal-volume mechanical ventilation (Kompass, 2010; Wallace, 2009; Wu, 2008). Secondly, premature sheep exposed to chorioamnionitis have decreased levels of CTGF despite having elevated levels of TGF β and phosphorylated Smad2 (Kunzmann, 2007), suggesting pathways other than TGF β influence its expression. CTGF can activate Wnt signaling pathways, reciprocally enhance TGF β signaling and inhibit VEGF, which are all known to play roles in postnatal lung development. Importantly, CTGF expression was shown to be dramatically increased in lungs of children that died with BPD (Alapati, 2011), making it an obvious candidate in the pathophysiology of BPD.

Like many growth factors in lung development, the level of CTGF expression must be tightly controlled to achieve normal alveolarization. Over-expression of CTGF in the conducting airway or distal airspace epithelium from P1 to P14 durably inhibited secondary septation and pulmonary vascular development by P7 (Chen, 2011; Wu, 2010). Lungs were fibrotic with increased muscularization of the pulmonary vasculature resulting in signs of pulmonary hypertension. In addition, there was evidence that CTGF activated a pulmonary inflammatory response with influx of polymorphonuclear neutrophils and macrophages and elevation of pro-inflammatory cytokines. Furthermore, in a hyperoxic murine model of BPD, CTGF-neutralizing antibody given throughout hyperoxic exposure improved alveolar and vascular development and attenuated pulmonary hypertension (Alapati, 2011). Although excessive levels of CTGF are detrimental to alveolar development, genetic ablation of CTGF causes lung hypoplasia and immediate neonatal death due to abnormal musculoskeletal development (Baguma-Nibasheka and Kablar, 2008), suggesting that there may be a minimum requirement of CTGF during lung morphogenesis.

Vascular Endothelial Growth Factor (VEGF)

VEGF is a potent vascular mitogen that is normally expressed in the alveolar epithelium of the developing lung with peak expression occurring at the time of alveolarization (Ng,

2001). Inhibition of VEGF signaling pathways has been demonstrated in infants dying of BPD (Bhatt, 2001; Lassus, 1999; Lassus, 2001), and diminished tracheal aspirate levels from ventilated infants on the first postnatal day can predict the subsequent risk for BPD (Been, 2010). Animal models utilizing hyperoxia (Balasubramaniam, 2007; Kunig, 2005; Tang, 2010; Thebaud, 2005), endotoxin exposure (Tang, 2010), or mechanical ventilation (Mokres, 2010) to induce BPD display inhibited VEGF signaling concomitant with disrupted alveolar and vascular development. Pharmacologic inhibition of the crucial VEGF receptor, VEGF-R2, in neonatal rats inhibits not only vascular but also alveolar development (Jakkula, 2000; Le Cras, 2002), supporting VEGF's vital role in pulmonary microvascular and alveolar development. Subsequent studies have shown attenuation of hyperoxia-induced BPD with VEGF supplementation (Kunig, 2005; Thebaud, 2005), further strengthening the notion that VEGF-mediated angiogenesis is vital for normal lung development.

VEGF signaling appears to support lung development via the production of nitric oxide (NO) by the pulmonary endothelium, as supplementation with inhaled NO (iNO) attenuates the arrested alveolar and vascular development that results from VEGF inhibition (Bland, 2005; Lin, 2005; McCurnin, 2005; McElroy, 1997; Tang, 2004; Tang, 2007; ter Horst, 2007). Therefore, several randomized controlled trials have been conducted to determine if iNO reduces the incidence of BPD when used at an early age. Although evidence from observational studies and animal models suggested that iNO would restore VEGF signaling pathways and reduce the incidence of BPD, both a recent meta-analysis (Donohue, 2011) and an NIH consensus panel review (Cole, 2011) of RCTs conducted to date failed to show a significant effect for the majority of infants. Animal studies are surfacing that utilize the phosphodiesterase-5 inhibitor, Sildenafil, as an alternative means to augment the iNO pathway via the decreased degradation of the essential signaling molecule, cyclic GMP (de Visser, 2009; Ladha, 2005). As iNO can only be reliably administered via an endotracheal tube and mechanical ventilation is now largely avoided in many extremely preterm infants, a non-inhalational therapy like Sildenafil may extend the pro-angiogenic benefits of the iNO pathway to a larger proportion of infants at risk for BPD. Clinical studies assessing the feasibility, safety, and efficacy of Sildenafil administration to extremely preterm infant are lacking, however, and its routine use cannot be recommended at this time.

Future Directions

Although extensive knowledge exists for the mechanisms responsible for early lung development (Kimura and Deutsch, 2007; Morrissey and Hogan, 2009; Warburton, 2000), the mechanisms governing alveolarization are only just beginning to be resolved and remain poorly understood. Compounding the difficulty of unraveling the complex network of signaling that must occur for alveolar lung remodeling to proceed normally, we are hindered by a lack of adequate human specimens to reliably characterize human BPD as current animal models may not adequately recapitulate the human condition. Gene array studies that identify shifts in gene expression during normal and aberrant alveolarization are beginning to emerge (Boucherat, 2007; De Paepe, 2010; Foster, 2006; Mariani, 2002; Wagenaar, 2004), and will likely identify new candidates participating in alveolar septation. Attention to the genes that are expressed within and adjacent to the growing septal tips will likely yield the greatest information regarding septal initiation, elongation, and maturation (Foster, 2006). One such study has identified Wnt5a, NDP, FZD1, and Hox5a as proteins that localize to the growing septal tips and are up-regulated from P2 to P7, the period of early murine alveolarization (Boucherat, 2007). While the function of these genes in lung development remains unknown, they are the beginning of an exciting trend to identify much needed novel molecular markers to begin to unravel the underlying pathophysiology of BPD in animal models and optimistically verify within patient specimens.

Importantly, we must utilize our knowledge of significant time-points in alveolar septation to design appropriate studies utilizing gene array and modern genomics tools. There appears to be a critical window in distal airspace development where the lung is particularly susceptible to injury. As stated above, the majority of infants that develop BPD are born during the period of early saccular lung development, suggesting that aberrant development of terminal airway branches is sufficient to impair ultimate lung structure and function. Accordingly, neonatal mice exposed to 60–100% O₂ during postnatal saccular lung development (P0 to P4) and then allowed to recover in room air have persistent functional and structural deficits in young adulthood (4–8 weeks), the severity of which correlate to increasing levels of O₂ exposure (O'Reilly, 2008; Yee, 2009; Yee, 2006). In a follow-up study, mice similarly exposed to 100% O₂ from P0–P4 eventually had recovery of lung architecture by 67 weeks, but abnormalities in vascular development and pulmonary function remained (Yee, 2011). The observations from these studies would suggest that, although the developing lung retains the ability to partially recover from early insults, lung injury during the period of saccular development is sufficient to phenocopy BPD. An understanding of the mechanisms involved in lung recovery following early, brief injury is, therefore, important to determine how we utilize therapies to modulate lung development following preterm birth to avoid BPD. Secondly, while the bulk of murine alveolarization from immature septa occurs from P4 to P15, there is also a considerable amount of new septa that originate from mature alveolar walls between P14 and P36 (Schittny, 2008). This latter period of septation could be most important to preterm infants, as it may represent an opportunity for “catch-up” septal growth to acquire additional alveolar surface area when the initial insults incurred in the NICU have passed. Therefore, it may be prudent to identify the changes in gene expression that occur as animals are recovered from the inciting exposures that inhibited their early alveolar development rather than the alterations in the acute phase of injury.

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FIGURE 1. Disordered alveolar development in BPD

(A) Normal alveolar development begins with the initiation of secondary septa at sites of elastin deposition within the primary septa. Note the double capillary network (red circles) within the saccular walls. (B) As the saccular airspace undergoes normal alveolarization, it thins and a single capillary network emerges in close opposition to the air interface. Secondary septa elongate and have elastin localized to their tips. (C) In BPD, blunted secondary septa are present along with aberrant, disorganized elastin depositions in the saccular wall. The pulmonary microvasculature is underdeveloped and located within thickened septal walls.

Table 1

Mesenchymal proteins and growth factors implicated in the pathogenesis of BPD.

Candidate	Expression in distal lung development	Expression in models of BPD	Effect of over-expression/supplementation	Effect of ablation/inhibition	Clinical correlate
Elastin	Tips of developing secondary septa	Up-regulated but disordered (MV, O2)	N/A	<i>Systemic KO</i> : Inhibited alveolarization despite normal saccular lungs	Increased but disorganized in autopsy specimens of infants that died with BPD
PDGF	α SMA myofibroblasts (-R α) in growing septal tips	Decreased (O2)	N/A	<i>Systemic PDGFA KO</i> : Inhibited alveolarization with loss of α -SMA+ myofibroblasts <i>Inhibitory PDGF-Rα Ab (P1-7)</i> : Inhibited alveolarization	Clinical correlates lacking
FGFR-2,3,4	Alveolar Epithelium-peaks in alveolarization	-R3,4: Decreased, then increased (O2)	N/A	<i>Soluble DN (-R2)</i> : Inhibited alveolarization (canalicular); no effect (alveolar) <i>Compound Systemic KO (-R3, -4)</i> : Normal saccular but failed alveolar development	Clinical correlates lacking
FGF-7	Alveolar Epithelium, microvascular mesenchyme	Increased, then decreased (O2, MV)	Enhances cell survival without rescue of alveolar septation defect	<i>Neutralizing Ab</i> : Inhibited proliferation, vascular and alveolar development when given postnatal	Levels at d5 indirectly proportional to BPD risk
FGF-10	ECM beneath alveolar epithelium	Decreased (LPS)	Reversed LPS-induced inhibition of saccular airway development	<i>Systemic KO</i> : Die at birth due to complete lack of lung development	Reduced in autopsy specimens of infants that died with BPD
TGFβ	Alveolar epithelium and ECM	Increased (O2, MV)	Postnatal transgenic or adenoviral overexpression: Inhibits alveolarization Prenatal genetic over-expression: Arrest in saccular lung development	<i>Neutralizing Ab (Tgfb-1,-2,3)</i> : Attenuated hyperoxia-induced inhibition of alveolarization <i>Systemic KO (Smad 3)</i> : Inhibited alveolarization evident at P7 <i>Lung epithelium-specific TBR-II KO</i> : Inhibited alveolarization by P7	Elevated tracheal aspirate levels predict BPD risk/O2 dependence
CTGF	Epithelium and then mesenchyme of terminal airways; intensifies in late gestation and then diminishes just prior to birth	Increased (O2, MV) Decreased (ETX)	Inhibited alveolarization by P7 when over-expressed in P1-14	<i>Systemic KO</i> : Lung hypoplasia <i>Neutralizing Ab</i> : Attenuated hyperoxia-induced inhibition of alveolarization	Increased in autopsy samples of infants that die with BPD
TGF-α	Mesenchymal cells surrounding distal saccular airspaces; Saccular epithelium	Increased (O2)	Inhibited alveolarization when over-expressed in late canalicular/early saccular (E16.5-18.5) or when over-expressed at beginning of alveolarization (P3-5)	<i>Systemic KO (EGF-R)</i> : Defective airway branching by E12	Elevated levels in lungs of infants that died of BPD
VEGF	Expression peaks during alveolarization in alveolar epithelium (Type-II Cells)	Decreased (O2, ETX)	Attenuated hyperoxia-induced inhibition of alveolarization	<i>Neutralizing Ab</i> : Inhibits vascular and alveolar development	Infants that die with BPD have reduced VEGF as well as its downstream protein products in lungs

(MV: mechanical ventilation, oxygen: O2, ETX: endotoxin, Ab: Antibody, α SMA: smooth muscle actin, KO: knock out, LPS: lipopolysaccharide, DN: dominant negative)