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Differential Expression of Angiogenic Gene Networks during Post-natal Lung Alveolarization

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Abstract

Prematurity of birth is the leading cause of death in children under 5 years of age. Preterm birth rates are steadily increasing across the globe due to advancements in medical knowledge and procedures that allow progressively earlier fetuses to survive outside of the mother's womb. Bronchopulmonary dysplasia is a chronic lung disorder that can result from mechanical ventilation and long-term oxygen use in preterm neonates. This condition is characterized by disrupted pulmonary alveolar septation, alveolar hypoplasia, and fewer, larger alveoli that lead to decreased surface area available for gas exchange. Despite notable advances in preterm care, bronchopulmonary dysplasia results in a large number of short- and long-term morbidities, thus more advanced treatments that facilitate lung development in this subset of pediatric patients are sorely needed. In this study, we examined alterations in the gene expression profiles of 81 genes involved in angiogenesis during the process of normal alveolarization in mouse lungs. Our data revealed that alveolarization can be largely divided into early (1 week postnatal) and late (4 to 8 weeks postnatal) stages based on angiogenic gene expression profiles. In summary, our findings provide molecular-level data illustrating the dynamic alterations in pulmonary angiogenesis during postnatal alveolarization. This knowledge can be used to provide a better understanding of normal lung development, and set the stage for discovering targeted therapies that can assist in pulmonary functioning for preterm infants.

Keywords: Cerebral palsy; Bronchopulmonary dysplasia; Alveolarization; Angiogenic regulators

Introduction

Preterm birth affects 1 of every 10 infants born worldwide and is the greatest contributor to infant death [1]. The fetus undergoes critical developmental changes in the final weeks of gestation, and disruption of these processes has been associated with breathing problems, feeding difficulties, cerebral palsy, developmental delay, vision problems, and hearing impairment. One of the most common pulmonary conditions associated with premature delivery is bronchopulmonary dysplasia (BPD), which is a chronic lung disorder that can affect pre-term infants, especially those that require prolonged mechanical ventilation to treat respiratory distress syndrome (RDS) [2]. BPD is caused by prolonged exposure to high oxygen in premature infants, which results in necrotizing bronchiolitis, alveolar septal injury, abnormal vascular growth, and scarring, ultimately leading to hypoxemia due to impaired pulmonary function. According to the National Heart, Lung, and Blood Institute, there are between 5,000 and 10,000 cases of BPD every year in the United States. Babies with extremely low birth weight (less than 1000 grams) are most at risk for BPD, and though many of the symptoms are severe, most patients will outgrow the more serious symptoms [3,4]. Decreased expiratory volume and abnormal heart enlargement have been associated with long-term effects of BPD [3,4].

Lung development is divided into four histological stages which include pseudoglandular (human 5-17 weeks gestation; mouse E9.5-E16.6), canalicular (human 16-25 weeks gestation; mouse E16.6-E17.4), saccular (human 24 weeks to late fetal period; mouse E17.4-P5), and alveolar (human late fetal period to childhood; mouse PN5-PN30) stages [5-7]. In the alveolar stage, mature alveolar ducts and alveoli are generated through a process called alveolarization. In neonatal humans, roughly one third to one half of the approximately 300 million alveoli are formed, and within the first six months of post-natal development the number of alveoli increases substantially with the adult number being reached by three years of age. BPD leads to disturbances in alveolarization and pulmonary vasculature development, resulting in lungs with fewer and larger alveoli, a dysmorphic pulmonary vasculature, reduced capacity for gas exchange, and increased morbidity/mortality.

Significant advances in our understanding of lung development have been reported; however most of these studies have focused on prenatal lung development, and far less is known regarding postnatal lung development. Understanding the regulatory networks that coordinate alveolar and vessel formation in late stage lung development will provide critical knowledge that not only will deliver an understanding of normal pulmonary function, but also may help explain responses to pathophysiological stimuli such as BPD and serve as the stepping stone to develop treatments for this condition.

The alveolar region of the lungs is efficient for gas exchange only if there is sufficient blood flow to the area, thus pulmonary vessels run parallel to the airways to facilitate this process. Because of the close association between blood vessels and airways, it is likely that common factors may contribute to their mutual formation. In this study, we sought to understand the angiogenic gene regulatory networks involved in alveolarization using a mouse model system. To accomplish this, we employed a genomics/bioinformatics approach to monitor the gene expression of angiogenic regulators at defined time-

points in postnatal mice at 1, 4, and 8 weeks old, corresponding to early, middle, and late alveolarization in humans.

Materials and Methods

Microarray dataset. Our meta-analysis was based on a previous study carried out by Finkielstain et al. using microarray based whole genome expression analysis on lung tissue collected from 1 (N=5), 4 (N=5), and 8 week (N=5) old mice [8]. This data was deposited in Gene Expression Omnibus (GEO# GDS4316).

Heatmap generation. Tab-delimited files of the gene expression data obtained from GEO# GDS4316 were imported into Cluster 3.0 [9]. The data was normalized and both the genes and arrays were subjected to unsupervised hierarchical clustering using a centroid linkage. Heatmaps were visualized using Java Tree view software [10].

Results

Finkielstain et al. previously performed microarray based whole genome expression analysis on lung tissue collected from 1 (N=5), 4 (N=5), and 8 week (N=5) old mice [8]. This data was freely deposited in Gene Expression Omnibus (GEO# GDS4316), allowing bioinformatics meta-analysis of the dataset.

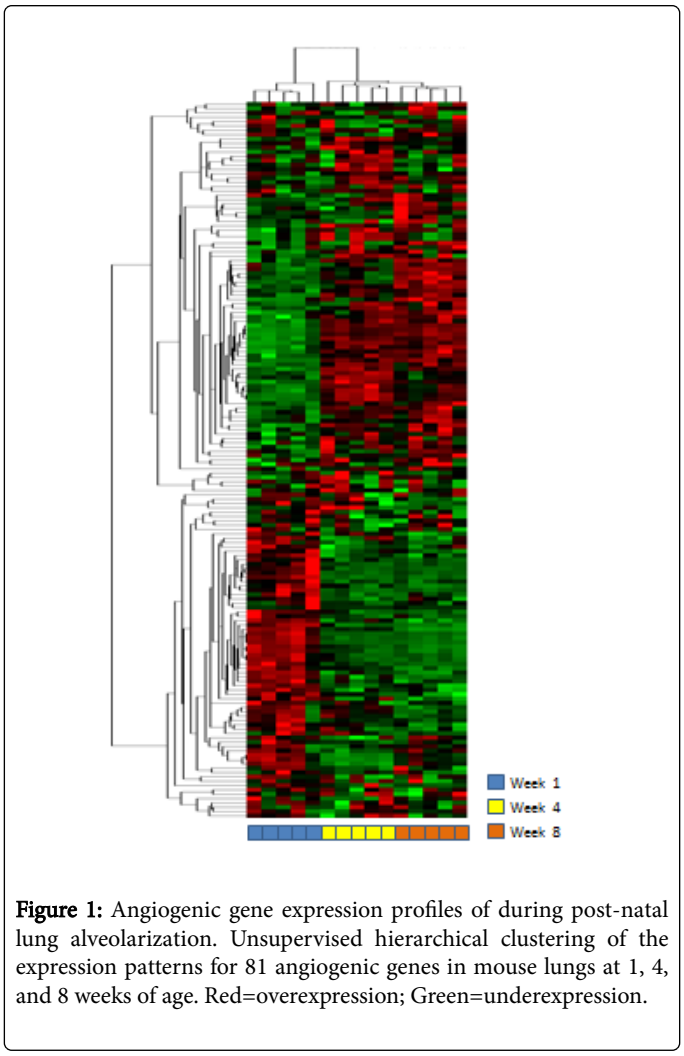

Figure 1: Angiogenic gene expression profiles of during post-natal lung alveolarization. Unsupervised hierarchical clustering of the expression patterns for 81 angiogenic genes in mouse lungs at 1, 4, and 8 weeks of age. Red=overexpression; Green=underexpression.

Using a targeted approach, we examined the gene expression patterns of 81 genes and their isoforms with strong reported evidence supporting their involvement in vascular processes such as angiogenesis, vasculogenesis, and vessel remodeling.

Unsupervised hierarchical clustering analysis revealed perfect clustering of the lung tissues according to developmental stage (Figure 1), suggesting that calculated modulation of genes involved in vascular processes is associated with the process of alveolarization. Clustering patterns on the heatmap revealed an obvious trend in the gene expression profiles that differentiated the lungs of 1 week old mice (early alveolarization) from that of 4 and 8 week old mice (middle and late alveolarization) (Figure 1).

While the vascular gene expression patterns between weeks 4 and 8 were largely similar, there were a small handful of genes that were differentially expressed between these time-points, suggesting that the process of pulmonary vascular development slows, but does not fully cease in late alveolarization (Figure 1).

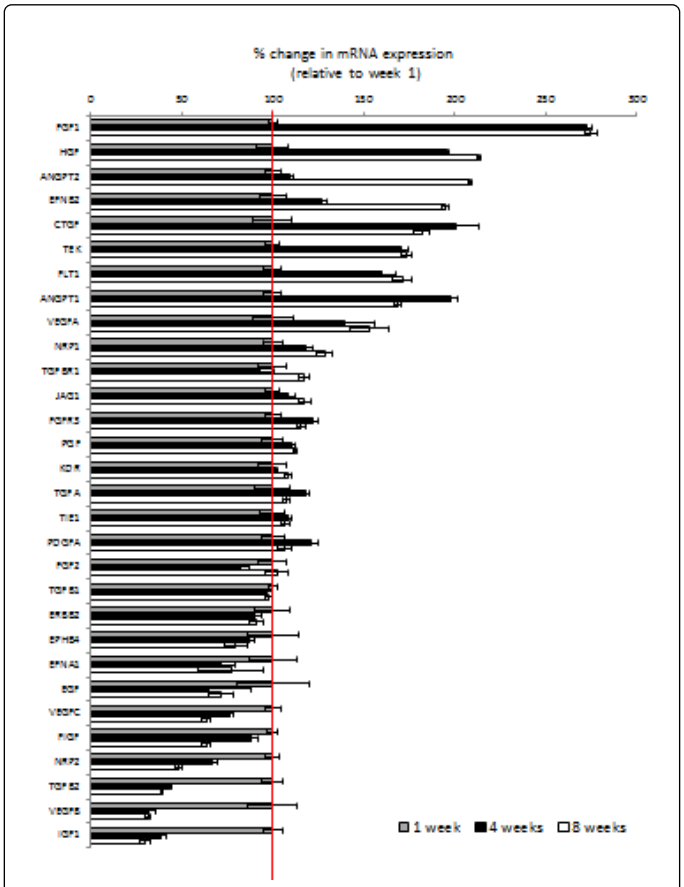

Figure 2: P Genes involved in angiogenic growth factor signaling are differentially regulated during post-natal lung alveolarization. Histogram depicting the expression profiles of selected angiogenic growth factor signaling genes in mouse lungs at 1, 4, and 8 weeks of age. The red line indicates the baseline expression of the genes at week 1.

Figures 2-5 reveal the relative expression patterns of genes involved in angiogenic growth factor signaling, extracellular matrix remodeling,

inflammation, and associated processes, respectively, in postnatal weeks 1, 4, and 8.

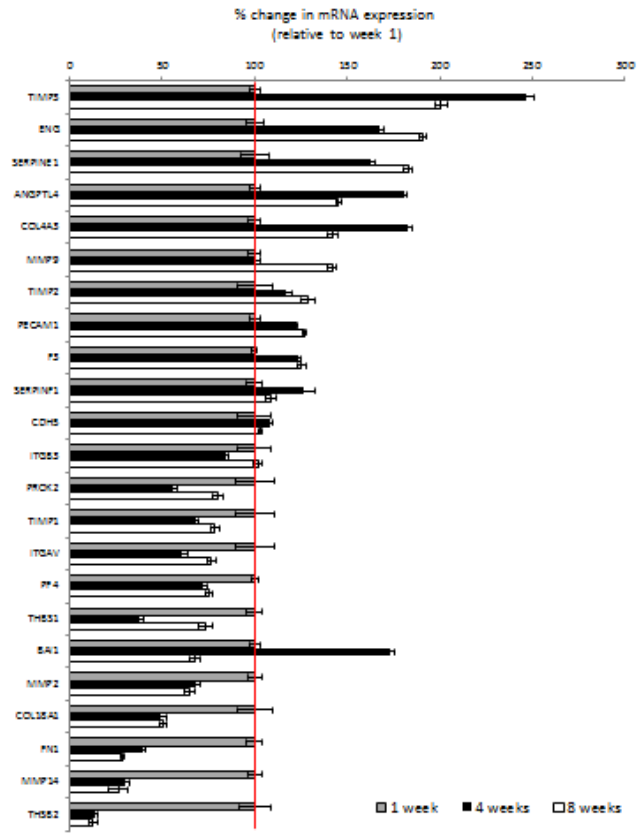

Figure 3: Genes involved in extracellular matrix remodelling are differentially regulated during post-natal lung alveolarization. Histogram depicting the expression profiles of selected extracellular matrix remodelling genes in mouse lungs at 1, 4, and 8 weeks of age. The red line indicates the baseline expression of the genes at week 1.

Discussion

Our data revealed that expression of angiogenic gene regulatory networks can be largely divided into early (postnatal 1 week) and middle/late (postnatal 4-8 weeks) alveolarization. Our findings uncovered significant alterations in the expression of genes involved in angiogenic growth factor signaling, extracellular matrix remodeling, inflammation, and other processes. These findings are important because it is critical to clearly understand normal postnatal lung development processes in the hope that future studies can extrapolate strategies to treat pulmonary dysfunction such as that found in BPD.

Similar bimodal staging of alveolarization has been reported in the literature. For example, Mund et al. uncovered two phases of developmental alveolarization using a mouse model, with the process divided into a P4-P21 stage where new septa are formed from immature pre-existing septa, and a second phase starting at P14 and continuing into young adulthood where new septa are formed from mature pre-existing septa [6].

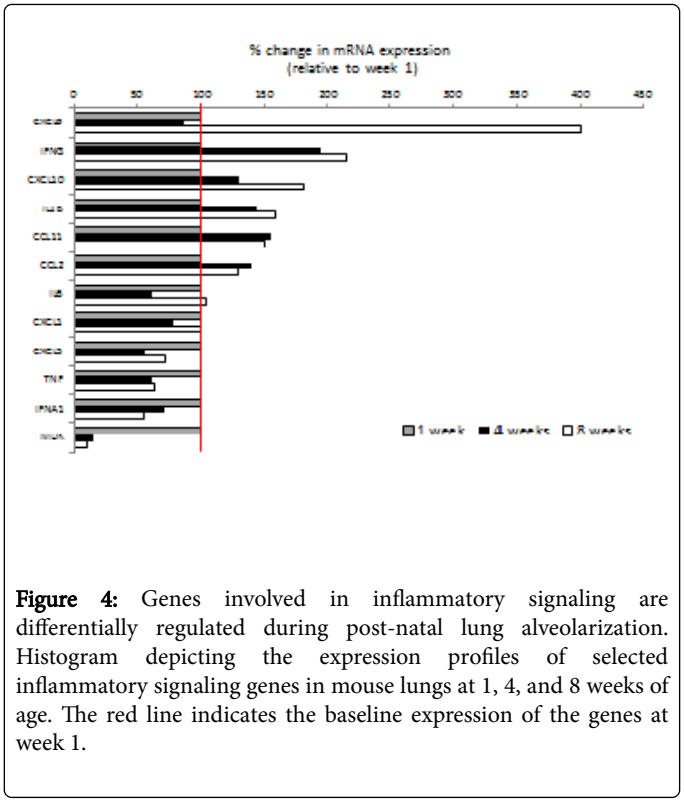

Figure 4: Genes involved in inflammatory signaling are differentially regulated during post-natal lung alveolarization. Histogram depicting the expression profiles of selected inflammatory signaling genes in mouse lungs at 1, 4, and 8 weeks of age. The red line indicates the baseline expression of the genes at week 1.

A study by Finkielstain et al. examining gene expression alterations that occur during slowing of postnatal organ growth revealed 3531 upregulated genes and 6535 downregulated genes changing with age in mouse lung development [8]. This study implicated expression changes for genes such as Peg3, Mest, and Igf2 in postnatal somatic growth deceleration. While other studies have examined the expression and contribution of individual genes or gene families in postnatal alveolarization, a network level approach to understand gene expression during this process has not been reported in the literature. Our data demonstrated that genes encoding angiogenic growth factors such as FGF1, HGF, ANGPT2, CTGF, VEGFA and their receptors EFNB2, TEK, and FLT1 were upregulated in lungs of 8 week old mice relative to that of 1 week old mice. Similarly, downregulation of EGF, FIGF, IGF1, NRP2, VEGFB, and VEGFB was observed in 8 week old mice. Interestingly, dysregulation of many of these genes is reported for BPD and other pediatric pulmonary conditions. For instance, short-term ventilation of lungs stimulated a transition from traditional angiogenic growth factor expression to that of alternative anti-sprouting regulators, and this shift has been suggested to contribute to deficient alveolarization characteristic of infants with BPD [11].

Artificial addition of factors such as HGF and EGF through pharmacological modulation or adenoviral expression, or via modulation of VEGF levels through HIF1-alpha overexpression, has been shown to improve alveolarization and other pulmonary output characteristics in models of BPD [12-14]. Moreover, an LRP5-Tie2-Ang signaling axis has been shown to play a central role in angiogenesis and alveolarization during postnatal lung development, and disruption of these pathways leads to abnormal lung phenotypes similar to that exhibited in patients with BPD [15]. Finally, infants with BPD exhibited increased IGF1 levels compared to normal preterm or term infants, and this reduction was due to BPD-associated increases

in epithelial miR-489 that is secreted in exosomes and targets IGF1 on its 3' untranslated regions on fibroblasts [16].

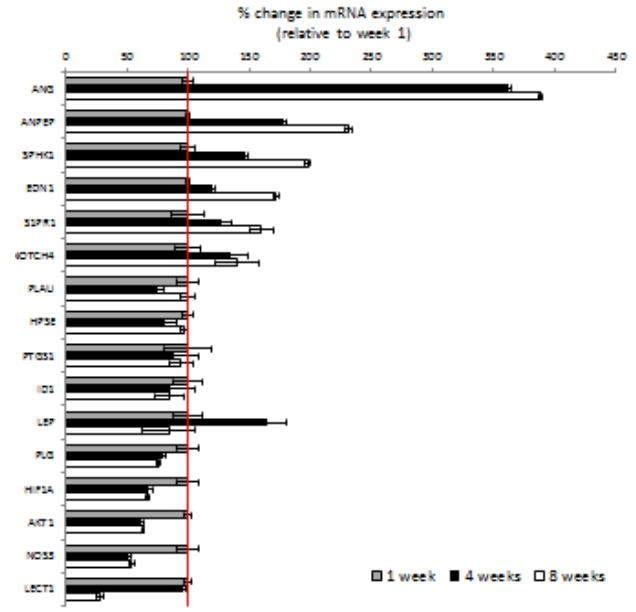

Figure 5: Genes peripherally involved in angiogenic processes are differentially regulated during post-natal lung alveolarization. Histogram depicting the expression profiles of genes peripherally involved in angiogenic processes in mouse lungs at 1, 4, and 8 weeks of age. The red line indicates the baseline expression of the genes at week 1.

Our data demonstrated that genes encoding extracellular matrix remodeling factors such as MMP9 and its inhibitors TIMP2 and TIMP3 were upregulated in lungs of 8 week old mice relative to that of 1 week old mice. Similarly, downregulation of MMP2 and MMP14 and their regulator TIMP1 was observed in 8 week old mice. Previous studies concur with our data, reporting distinct MMP/TIMP expression patterns in pulmonary mesenchymal and epithelial cells, with adult lungs exhibiting a more anti-proteolytic profile reflective of decreased MMP2 and increased TIMP2/3 expression [17,18]. Extensive remodeling of the lung parenchyma has been reported in pulmonary disorders such as BPD and associated with re-epithelialization of the alveoli and fibrotic formation. Indeed, aberrant regulation of MMPs and TIMPs is strongly correlated to decreased collagen turnover in BPD fibrosis [19]. Low levels of MMP2 and higher MMP3, MMP9, and TIMP2 at birth were predictive biomarkers indicating increased risk of BPD [20-22]. Exposure of neonatal mice to chronic hypoxia resulted in upregulation of MMP2 and downregulation of TIMP2, while hyperoxia decreased MMP9 and TIMP1 activity [18]. Gene deletion or pharmacological inhibition of MMP2, as well as administration of a pan-MMP inhibitor resulted in abnormal pulmonary arterial remodeling and impaired alveolarization in mouse models [23,24]. Finally, certain MMP16 polymorphisms have been found to protect human neonates from BPD [25].

Our data demonstrated that genes encoding inflammatory factors such as CCL2, CLL12, CXCL9, CXCL10, IFNG, and IL1B were up regulated in lungs of 8 week old mice relative to that of 1 week old mice. Similarly, down regulation of CXCL5, IFNA1, and TNF was observed in 8 week old mice. Significant evidence links regulation of NFKB inflammatory and angiogenic signaling with postnatal lung development [26]. Indeed, the inflammatory genes identified in our study are direct targets of the NFKB transcriptional complex. The inflammatory response of BPD is characterized by an accumulation of neutrophils, macrophages, and increased pro inflammatory mediators that alter endothelial and alveolar integrity [27]. Increased concentrations of CXCL10 and CXCL11 are reported in BPD affected lungs [28], and the ratio of IL1beta to IL1Ra in tracheal aspirates from preterm infants with respiratory failure has been shown to be predictive BPD development [29]. TNF-alpha immunoreactive alveolar and interstitial macrophages are present in large numbers in BPD patients, aberrant TNF-alpha levels have been correlated to increased incidence of BPD, and inhibition of TNF-alpha with pharmacological antagonists prevented pulmonary hypertension [30-32].

Conclusion

In conclusion, we have identified a number of angiogenic factors that are differentially regulated at the gene expression level during postnatal lung development. Dysregulation of many of these factors has been shown to play an important role in the pathogenesis of BPD. Further studies should be carried out to determine if modulation of these gene networks can lessen the severity of neonatal pulmonary disorders such as BPD, and ultimately decrease pediatric morbidity and mortality.

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