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## Perinatal Lung Development: The Lung at Birth

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Additional information is available at the end of the chapter

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### Abstract

The successful transition from liquid to air breathing at birth is essential in mammalian lung development and the primary biological role of the hypothalamic-pituitary-adrenal axis. At this moment, the lung experiences a major environmental change in oxygen tension as the fluid that filled this organ in utero ( $pO_2 \sim 26$ ) is rapidly replaced with ambient air during the first few breaths ( $pO_2 \sim 150$ ). This change induces oxidant stress in the lung which is balanced by antioxidant genes that are induced in the late gestational fetal lung to protect against injury. These antioxidant genes impact distinct antioxidant molecules, including glutathione, which can regulate the level of redox stress in lung cells. Cells, such as the alveolar macrophage, are central for host defense and surfactant homeostasis at the newly established air-liquid surface. Yet they are prone to dysfunction with excessive oxidant stress and the newborn lung is susceptible to infection. And yet the rise in alveolar oxygen tension can also serve as a physiologic redox signal that initiates expression of genes that regulate postnatal lung development. Taken together, birth can be viewed as a natural experiment with hyperoxia where the birth process itself serves as an integrator for that level of redox stress that limits lung injury while activating genes required for postnatal lung development.

**Keywords:** lung, perinatal, birth shock, glutathione, Kruppel-like factor 4

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### 1. Introduction

The symbols for man (♂), woman (♀), birth (✳), death (†), and infinity (∞) were penned on a blackboard at the opening of the 1960s medical drama “Ben Casey.” These symbols represented a fundamental organization of life in the universe of medicine. Birth was a culmina-

tion of a progressive series of molecular and cellular events that began with fertilization, progressed through gastrulation, and then embryogenesis to form the fetus and finally the newborn child. Along the way, an ever enlarging population of cells proliferated, migrated, differentiated, and died and a host of organs with unique functions were constructed on organ-specific extracellular matrices connected to each other by vessels and to the external environment by tubes. Herein, we focus on a select aspect of lung development in the perinatal period. At that time, the fetus experienced a unique transition from a highly protected fluid-filled uterine environment to a highly susceptible gas-filled environment that marked its independent existence as a newborn. The role of the lung as a secretory organ came to an end and its new role as a gas exchanger began. In so doing, the cells of the lung, more so than any other organ, were immediately exposed to an elevated concentration of oxygen.

Decades of lung developmental research have unraveled many features about lung function in the perinatal period. Some highlights include a primary role for hormones in regulating lung cell maturation along with the ontogeny of a surfactant lipoglycoprotein complex to ensure the success of this transition, particularly the hypothalamic-pituitary-adrenal axis [1]. Although some aspects of perinatal lung alveolar, cell surface differentiation is steroid-independent [2]. Identification of regulators that control the progression of lung progenitor cells into distinct lineages to form distal lung alveolar cells [3]. The molecular events occurring during lung development are regulated by transcription factors, and the families involved are extensive [3, 4]. In this review, we will focus on a particular transcription factor family known as the Kruppel-like factors (KLF). Krüppel is a zinc-finger transcription factor described in *Drosophila melanogaster* as essential for pattern formation and embryogenesis [5]. A related family of mammalian Krüppel-like factors, Klf, share sequence homology with the DNA-binding domain of Krüppel and serve essential roles in mammalian embryogenesis [6]. Lung Kruppel-like factor, Klf2, has been known to be important in lung development [7]. Another member, Klf5, was shown more recently to play a role in perinatal lung development [8]. Interestingly, Klf5 is known to interact with Klf4, gut enriched Kruppel-like factor, which we will highlight in this review [9].

## 2. The Kruppel-like family of transcription factors and the lung

Lung development is characterized by a dramatic structural and functional re-organization from an organ of secretion *in utero* to one of the gas exchanges at birth. This morphologic transition is only partially complete in the newborn murine and human lung. The process of forming new alveoli in the lung occurs largely in the postnatal period. It involves regulation of cell proliferation, differentiation, migration, and apoptosis and affects epithelial, endothelial, and interstitial cell populations within the lung parenchyma and airways. It also involves complex interactions between cells and extracellular matrix proteins [10]. Previous literature described many of the cellular components of these critical postnatal events, and contemporary research has concentrated on the molecular regulators that drive the formation of the gas exchange surface and the mechanical properties of the postnatal lung.

It was known that there was a progressive decline in cell proliferation in mesenchymal cells, including fibroblasts [11] and epithelial cells [12, 13], near the end of gestation, at birth, and during the early postnatal period. The effect was transient as the number of proliferating cells rose dramatically in the late postnatal period with the onset of alveolarization and continued over the next 2–3 weeks [14]. During this time, the interstitium, which was thick and packed with cells at birth, became progressively more thin and sparse with maturation of the alveolar wall [15]. The relative number of interstitial cells actually declined.

Fibroblast differentiation after birth was believed to serve major function in forming alveoli in the late postnatal lung. A population of myofibroblasts, located at the tips of developing septa, expressed  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and produced matrix proteins such as elastin. The location of these cells denoted sites of newly forming alveoli and alveolarization extended from postnatal day 5 through day 20 in the mouse lung. This ability to form new alveoli appeared to be dependent upon the response of the myofibroblast to certain stimuli such as FGF-3, FGF-4 [16], and PDGF-A [17] receptors, as well as deposited matrix protein [18, 19] and transforming growth factor (TGF)- $\beta$  activity, including a component of the TGF- $\beta$  signaling pathway, Smad3 [20, 21]. Myofibroblasts are believed to re-emerge *de novo* in adult lung following tissue injury [22] and are postulated to play a role in repair. Differentiated fibroblasts also produce matrix proteins, including elastin and collagen. The lung actually has very little connective tissue at birth. As a result, lung elastic recoil is limited and the lung is prone to rupture by applied pressure. Production of matrix proteins such as elastin and collagen by interstitial fibroblasts during alveolarization contributes to elasticity and structural integrity to the lung [23]. Interestingly, regulation of cell proliferation and cell differentiation is a role that is reiterated in much of the Kruppel family literature.

Overall, the Kruppel-like family currently consists of over 16 members, and the originally descriptive nomenclature is now being revised into a numerical grouping system based on sequence homology, functional domains and transcriptional activity [24]. For instance, Klf4 is also known as EZF (epithelial zinc finger) and gut-enriched Klf (GKLF). It activates and represses gene transcription and has autoregulatory activity [25]. Klf2 is also known as lung Klf. It is mainly an activator of transcription and plays a role in blood vessel and lung development. **Table 1** summarizes selected aspects of a select Klf s and more detailed summaries are available in recent reviews [24, 26–28].

The Klf s are a subset of a larger family that includes Sp1 and Sp1-like proteins with Sp1 being the founding member of the family. Sp1 and Sp-1 like proteins are grouped together by phylogenetic tree analysis of protein sequences (group I) and the Klf s divide into groups II and III [24]. In general, Sp1 and Sp1-like members preferentially recognize the GT box in promoters (5'-GGTGTGGGG-3'), whereas the Klf members preferentially recognize the CA box (5'-CACCC-3'). However, competition among members for these sites and post-translational modifications that affect DNA-binding site affinity is an active area of investigation. It is believed that this diversity of protein members allows for a precise regulation of gene expression under a variety of physiological conditions. The ability of the Klf family to regulate cell proliferation, survival, and differentiation of different cell lineages suggests a potentially broad role in normal development, response to injury, and carcinogenesis.

Klf #	Alternate name	Transcriptional activity	Cell functions	Phylogenetic grouping	Expression sites	Expressed in lung
Klf2	Lung KLF	Activator	Blood vessel, lung development, T cell survival	II	Blood vessels, hematopoietic cells	+ [29, 30]
Klf4	Gut KLF	Activator/repressor	Anti-proliferation Survival	II	Gut, epithelium, fibroblasts	+ [31, 32]
Klf5	BTEB2	Activator	Cell growth	II		+ [7, 33]
Klf6	COPEB	Activator	Putative tumor suppressor	II		+ [34]
Klf9	BTEB1	Activator/repressor	Neurite outgrowth Carcinogen metabolism	III	Ubiquitous	+ [35]
Klf13	BTEB3	Activator/repressor	Anti-proliferation Carcinogen metabolism	III		+ [36]

**Table 1.** Select characteristics for some members of the Krüppel-like family.

Klf4 was initially characterized in two independent laboratories. One identified it as a novel zinc-finger protein expressed in differentiated epithelial cells, hence named epithelial zinc finger (EZf) [31], and transiently in certain mesenchymal cells. The other identified it as a gut-enriched Krüppel-like factor expressed during growth arrest, hence GKLF [32]. In cultured fibroblasts, expression was abundant in growth-arrested cells and barely detectable in rapidly proliferating cells as the protein inhibited DNA synthesis. The message transcript was most abundant in colon followed by testis, lung, and small intestine by Northern blot and in epithelial cells of the colon as they migrated from the base to the top of the crypt [32]. Message was not detectable in the mouse embryo by Northern blot until day 15.5, but was evident in mesenchymal cells of the first branchial arch at day 11.5 and the metanephric kidney at day 12.5 by in situ hybridization. At day 15.5, the situ signal was detected in epithelial cells of the colon and the tongue and by the newborn period in epithelial cells of the esophagus and the stomach as well [31]. Message was also evident in skin of adult mice [31]. More recently, Klf4 expression has been defined in corneal epithelial cells [37]. This new gene was hypothesized to play a role in epithelial cells in many organs during differentiation in development and in mesenchymal cells during early formation of the skeleton and kidney.

These original studies demonstrated that Klf4 was an important regulator of cell proliferation and cell differentiation. In keeping with this concept, serum deprivation, DNA damage, and contact inhibition were associated with decreased proliferation and increased Klf4 expression [32, 38], while GI neoplastic lesions [39, 40] and cancer cell lines [41] were associated with high levels of proliferation but decreased levels of Klf4 expression. In tissues, Klf4 was expressed largely in post-mitotic, terminally differentiated epithelial cells [31, 32]. Transcriptional profiling of Klf4 confirmed this dual role in cell cycle regulation and epithelial cell differen-



tiation [42]. Cell cycle inhibitors were upregulated by Klf4, including the cdk inhibitor p21<sup>WAF1/CIP1</sup>, and cell cycle activators were downregulated, including cyclinD1 [38, 43]. Targeted elimination of the Klf4 gene in mice revealed its critical role in terminal differentiation of epithelial cells in the skin in the perinatal period [44]. Loss of barrier function was lethal within 24 h of birth due to the rapid loss of fluids. An independent Klf4 gene ablation experiment not only confirmed this observation but also showed an alteration in the terminal differentiation program of epithelial goblet cells in the colon [45].

In keeping with the known expression of Klf4 in mesenchymal cells, several studies demonstrated regulation of this gene in non-epithelial cell types. Human Klf4 was originally cloned from a human umbilical vein cDNA library after the initial description of mouse Klf4 [46]. More recently, Klf4 induction was observed in a genomic analysis of immediate/early responses to shear stress in human coronary artery endothelial cells [47]. Shear stress inhibits endothelial cell proliferation, and Klf4 was postulated to provide a mechanism for this effect. Studies with cardiac fibroblasts identified Klf4 induction by DNA microarray and linked its expression with several cell cycle and differentiation-associated genes that were induced with a sudden change from hypoxic to normoxic conditions *in vitro* to model “perceived hyperoxia” of ischemia-reperfusion. Klf4 was proposed to play a role in activating pathways for growth-arrest and fibroblast differentiation in these cells in response to oxygen-induced injury [48]. Klf4 has also been shown to mediate redox-sensitive inhibition of proliferation in vascular smooth muscle cells [49]. Klf4 induction in response oxidant exposure was dependent on hydroxyl radical production, intracellular calcium, p38 MAP kinase activation, and protein synthesis, although a transcriptional mechanism for its induction was not defined. The Klf4 response was rapid, detectable within 30 min, and associated with induction of p21<sup>WAF/Cip1</sup>, p27, and p53, known Klf4 target genes and inhibitors of cell proliferation. While Klf4 expression was known previously to respond to changes in the cell environment, this study identified oxidants as an additional extracellular signaling stimulus for Klf4 induction, and linked induction was activation of the MAPK signal transduction pathway.

Prior to our investigation, little was known about a role for Klf4 in lung development. Nonetheless, lung Klf4 expression had been reported in the lung in several different studies. The original characterizations of Klf4 noted expression in the adult [32] and the newborn lung [31]. Klf4 induction was also described in newborn compared to 4-week-old mice, a time of active lung cell proliferation and differentiation [50]. Early and transient Klf4 protein induction was described in the lung within 2 h following pneumonectomy although the cellular site was not determined [51]. Klf4 mRNA expression was induced in the airway transcriptome in response to cigarette smoke [52]. Klf4 mRNA was induced in alveolar epithelial type 1-like cells in response to ozone exposure [53]. Klf4 has very recently been shown to directly regulate TGF- $\beta$  induced myofibroblast differentiation in isolated adult rat lung fibroblasts by inhibiting expression of  $\alpha$ -SMA [54]. In this model, a Klf4 protein-protein interaction with Smad3 blocks Smad3 binding to a Smad3-binding element in the  $\alpha$ -SMA promoter. This direct linkage of Klf4 with Smad3 reveals a novel mechanism by which Klf4 regulates TGF- $\beta$ -induced myofibroblast gene expression in adult lung.

With this background in mind, our finding that lung *Klf4* mRNA was dramatically upregulated with birth in a normal oxygen environment and attenuation of this induction by birth in a more hypoxic environment led us to hypothesize that *Klf4* was regulated in a redox-responsive fashion in lung mesenchymal cells and that its target genes regulated fibroblast proliferation and differentiation events associated with postnatal lung development [55].

### 3. Oxygen as a regulator of Kruppel-like factor 4 in the newborn lung

This birth shock hypothesis was studied in time-pregnant mice by comparing findings in the lung with those in the liver, an organ that is not exposed to the same changes in environmental oxygen as the lung right at birth. And changes in newborn organs at 2, 6, 12, and 24 h after birth were compared with those of the corresponding fetal organ at day 21 of gestation, the time just before birth. Changes in gene expression at birth were examined with microarrays, and these data revealed an acute change in the level of expression of 157 genes within 2 h of birth in room air in the lung. The number of gene changes steadily declined at 6, 12, and 24 h thereafter. Most of the lung gene changes through 6 h involved transient induction or repression of expression. Less than 30% of the changes involved genes in common to any two successive time points, suggesting the presence of sequential waves of new gene expression over time. The full implication of this pattern is yet to be fully understood. Nonetheless, four functional categories of gene expression were overrepresented in these microarray data at 2 h after birth in room air: transcriptional regulators, structural genes, apoptosis-related genes, and antioxidants. Glutathione disulfide was analyzed to determine whether there was a change in the local redox state after birth in room air. This oxidation product accumulated fourfold within 2 h of birth and steadily decreased thereafter. Glutathione was always in excess of glutathione disulfide at all time points indicating a surfeit of antioxidant buffering capacity. But the lung is exposed to a transient oxidant stress and a change in the local redox state with the rise in environmental oxygen at birth.

Contrast these lung findings with those in the liver after birth in room air. The liver exhibited no accumulation of glutathione disulfide at the 2 h time point and no sign of oxidant stress at any of the early time points thereafter. These data suggest that the liver may not be subjected to the same magnitude of change in environmental oxygen tension as the lung at birth or it may follow a very different time course. In addition, there were far fewer changes in gene expression in the liver at 2 h after birth when compared to the lung, and no functional categories of gene expression were found to be overrepresented in the liver gene microarray dataset. There were a few gene expression levels that did change in common between these two organs. These could represent a shared response to the birth process itself. But we interpreted the majority of unique changes in the lung as a lung-specific response related to the change in alveolar oxygen content at birth.

To explore this, we examined a group of time-pregnant mice exposed to 10% oxygen using a glove bag at the 21st day of gestation. Some of these hypoxic mice were killed after 12 h to harvest fetal lung and assess baseline fetal measures at the end of gestation, whereas others were monitored and newborn lung was collected again at 2, 6, and 12 h after birth. Of note

here, all dams, fetuses, and newborns survived in the 10% oxygen environment, and this exposure did not significantly alter baseline gene expression in the fetal lung as determined with microarrays. This survival pattern suggested that changes in blood flow, lung stretch, nutrient supply, and fluid resorption were similar to that of mice birthed in room air and that these factors were less likely to differentially impact gene expression. Birth into 10% oxygen, however, did impact the level of redox stress in the lung at birth as well as the number of gene expression changes. Accumulation of glutathione disulfide was attenuated by half compared to the normal lung. And there was a 60% decrease in the number of gene changes at 2 h, compared to that in room air with elimination of any overrepresented functional category of gene expression. It was as if birth into an environment of hypoxia lacked any change in gene expression at all and retained a pattern like that of the fetal lung. The transient rise and fall in glutathione disulfide in the newborn lung in room air, despite the induction of several antioxidants in the late fetal lung and the lung at birth which buffered some but not all such stress [56–59], supports our hypothesis that a measured degree of redox stress, a birth shock, may serve a function in the lung at birth which we surmise is signalling expression of lung genes required for the onset of postnatal lung development [55].

The most provocative piece of data in our study was finding that the gene exhibiting the greatest change in expression in the lung at birth in room air was a transcription factor. And that transcription factor was Kruppel-like factor 4. Klf4 was previously referred to as gut Kruppel-like factor (GKLF), a tissue-specific gene that inhibited cell proliferation and stimulated differentiation in the gut, particularly in the epithelial cells of the crypts where there is a tight link between growth arrest and differentiation. Klf4 regulates cell proliferation by inhibiting cell-cycle progression and inducing growth arrest [31, 32, 60]. Mesenchymal and epithelial cell proliferation is known to progressively decline in the lung during the perinatal period [29–31], and a transcription factor like Klf4 is a reasonable candidate to regulate this process. Klf4 is known to induce growth arrest in cells through the cell-cycle inhibitor p21<sup>WAF/Cip1</sup>, and the absence of Klf4 in the Klf4 knockout mouse is associated with a corresponding decrease in the level of p21<sup>WAF/Cip1</sup> mRNA expression in the late fetal lung and persistent cell proliferation in fibroblasts and airway epithelial cells at birth [55].

Klf4 has previously been described in the mesenchyme during development [31]. We generated Klf4 knockout and Klf4 expressing fibroblasts to demonstrate that smooth muscle actin, fibronectin, tenascin C, and the alpha 1 chain of Type 1 collagen are Klf4 target genes and that fibroblast connective tissue gene expression is down-regulated in the perinatal lung of the Klf4 knockout mouse [55]. Impaired connective tissue gene expression has at least two distinct biological implications for postnatal lung development. First, lack of collagen synthesis decreases the tensile strength of the postnatal lung and increases the risk for rupture under pressure [61] and emphysema [62]. Second, lack of fibronectin, type 1 collagen, tenascin C, and smooth muscle actin expression can impair myofibroblast differentiation and postnatal alveogenesis [63–66]. Interestingly, Klf4 our study confirmed differential regulation of smooth muscle actin gene expression in the myofibroblast and the vascular smooth muscle cell as Klf4 deficiency in the Klf4 knockout mouse lead to a loss of expression in the myofibroblast (positive



regulator) but preservation of expression in the vascular smooth cell (negative regulator). This negative regulatory function in vascular smooth muscle cells was already known [67].

Finally, cell death was also increased in the Klf4-deficient lung of the Klf4 knockout mouse at birth. An expanding literature now describes Klf4 as an inhibitor of apoptosis [6]. Apoptosis is present even in the normal lung at birth and may play a physiologic role in postnatal lung development [68, 69]. We found an excess of apoptosis in the Klf4-deficient lung of the Klf4 knockout mouse, and this could well contribute to a loss of cell population and gene expression at birth. We also noticed that apoptosis exhibited a cell-specific rather than a general nature, as it was not increased in vascular smooth muscle cells within large blood vessels [55]. Taken together, we found good correlation between the gene categories that were overrepresented in the gene changes 2 h after birth and Klf4 as a potential regulator of these gene categories.

Is Klf4 expression in lung fibroblasts regulated by hyperoxia? We showed that it is and the mechanism is transcriptional in nature and independent of protein synthesis [55]. Further details of the mechanism await more studies. But Klf4 is known to be activated by oxidants, heat, chemical and mechanical forces, and nutrient deprivation [6]. Lack of Klf4 induction can be associated with stress [70] and the Klf4-deficient lung of the Klf4 knockout mouse at birth exhibited signs of stress such as increased degree of apoptosis and p53 expression and dramatic induction of p21<sup>CIP1/Waf1</sup> independent of Klf4. The full implications of Klf4 expression in the perinatal period will require further study in vitro using Klf4 deficient and sufficient cell cultures and tissue explants, but ultimate proof will require a fibroblast-specific conditional deletion of Klf4 in vivo [71, 72]. But our study was the first to draw attention to a physiologic role for oxygen as a regulator of this transcription factor for fibroblast and myofibroblast differentiation in normal postnatal lung development [55]. The role of Klf4 in regulating these fibroblast populations during lung development is likely to be recapitulated in the adult lung during repair of lung injury and to involve interactions with other Kruppel-like family members. The newborn lung at birth could provide a window into the responsiveness of an organism to changes in the environment of oxygen and oxidant stress.

#### **4. Glutathione in perinatal lung antioxidant defense and macrophage function**

In the alveolar space, alveolar macrophage (AM) surveillance is the first line of defense in the immune response as they ingest and clear pathogens, release cytokines and chemokines to recruit other immune cells, serve as antigen presenting cells, and interact with other alveolar cells to fine-tune the immune response. In order to fulfill their roles as sensors, transmitters, and responders to inflammation, AMs respond to endogenous and exogenous danger signals by changing their repertoire of surface receptor expression and functional phenotype to promote either host defense, wound healing, or immune regulation. This plasticity in their biological responses, including differentiation, phenotype, immune functions, and cellular interactions, is determined in large part by their extracellular milieu [73]. However, the phenotype is not permanent and pathogens or changes in the underlying microenvironment

can promote a change between a predominance of one set of characteristics to another phenotype. Depending on the different stimuli within the microenvironment within the alveolar space, alveolar macrophages are activated via distinct pathways that produce opposing effects on macrophage receptor expression, cytokine expression, and phagocytic function [74]. In response to interferon- $\gamma$  (IFN- $\gamma$ ) or microbial products, macrophages become classically activated (M1) macrophages and produce inflammatory cytokines and reactive intermediates of oxygen and nitrogen. In response to interleukin-4 or -13, macrophages become alternatively activated (M2) and produce anti-inflammatory mediators such as the interleukin-1 receptor antagonist and TGF- $\beta$ , promote tissue repair and remodeling, and become tolerant to stimulation by endotoxin in order to protect against overwhelming systemic inflammation. However, this immunosuppression is associated with a decreased capacity to clear microbes, and many diseases have abnormal shifts in the AM phenotype and immune responses that limit the ability of the cells to become innate immune effectors. Whether newborn AMs are skewed toward a M1 or M2 phenotype is unclear, but studies in a mouse model suggest that AM maturation in the fetal lung may include features of both pro-inflammatory and alternative activation paradigms [75].

Newborn infants, particularly those born preterm, face immunological challenges after birth because of the developmental stage of their immune systems. Upon birth, there is an age-dependent maturation of the intrapulmonary inflammatory responses with decreased AM immune responses such as phagocytosis and secretion of inflammatory mediators with potential immunoregulatory consequences [76, 77]. The functionally immature status of the newborn AM is suggested to be critical in the recurrent problem of early infancy pulmonary infections and morbidity [78]. At day 3 of life for premature newborns, the alveolar cells are more likely to have greater numbers of mononuclear phagocytes in the airway but fewer of these cells are functionally mature AMs [79]. This suggests that newborn AMs are functionally immature which may lead to increased susceptibility to lung infections. Furthermore, the degree of postnatal immune-suppression correlates with gestational age and is a predisposing factor for late infections [80].

The growth factor GM-CSF, granulocyte-macrophage colony stimulating factor, and its transcription factors PU.1 and Bach2 are increased after birth and necessary for AM differentiation and homeostasis making them critical to the AM immune response [81]. In newborn mice, the pleiotropic peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) modulates several transcription factors and genes associated with AM differentiation and immune functions. However, PPAR $\gamma$  expression in fetal lung monocytes is dependent on the GM-CSF pathway suggesting that GM-CSF has a lung-specific role in the perinatal development of AMs through the induction of PPAR $\gamma$  in fetal monocytes. Therefore, factors that suppress GM-CSF or PPAR $\gamma$  expression in the newborn AM also suppress differentiation and the development of a mature immune response as summarized in recent reviews [82–85].

In *in vivo* studies, ROS induces upregulation of TGF- $\beta$  expression [86], which is also a characteristic of a M2 phenotype [74]. Once TGF- $\beta$  is activated, it induces the GSH depletion and increases intracellular ROS production. Children with severe asthma have significantly higher concentration of TGF- $\beta$  in the epithelial lining fluid, which is associated with increased TGF-

$\beta$  and increased oxidative stress in the AMs [87]. In a mouse model, intranasal instillation of an adenovirus expressing constitutively active TGF- $\beta$  results in suppression of the expression of both catalytic and modifier subunits of glutamate-cysteine ligase (GCL), the rate-limiting enzyme in de novo glutathione synthesis, decreased glutathione concentration, and increased protein and lipid peroxidation in mouse lung [88]. Similarly, M2 macrophages have robust increases in TGF- $\beta$ 1 production and secretion [74], thereby creating a vicious cycle of ROS generation, TGF- $\beta$  production and signaling, and a M2 phenotype [89]. Indeed, mounting evidence suggests that AMs, particularly M2 AMs, are the predominant source of TGF- $\beta$  in lung fibrosis and depleting M2-polarized macrophages during the progressive phase of fibrosis reduces TGF- $\beta$  and collagen deposition [90]. In contrast, glutathione precursors induce proteolysis of the TGF- $\beta$  type II receptor and disintegration of TGF- $\beta$ 1 [86]. Therefore, perturbations in glutathione availability and its redox status in the epithelial lining fluid are important modulators of the AM phenotype and immune functions.

In addition a role for gestational development in the AM phenotype and immune response, the role of fetal exposures and subsequent changes in the microenvironment on the AM phenotype must also be considered. In animal models, *in utero* ethanol exposure also promotes oxidant stress in the newborn lung as evidenced by decreased concentrations of the reduced moiety and increased concentration of the oxidized glutathione moiety in the alveolus and neonatal AM [91]. This oxidation of the glutathione redox state in the alveolar space was associated with increased expression of TGF- $\beta$  and markers of a M2 phenotype in the newborn AM [92–94]. Impaired AM phagocytosis decreased the neonatal lung's defense against experimental Group B Strep as well as systemic sepsis [92]. However, addition of the glutathione precursor S-adenosyl-methionine (SAM-e) to the dam's diet restored the AM immune responses as well as decreased the experimental Group B Strep pneumonia and systemic sepsis in the newborn with fetal ethanol exposure. A central role for glutathione availability in the alveolar space in the AM immune functions was further supported by the ability of intranasal delivery of glutathione in the newborn to reverse the immune dysfunction associated with fetal ethanol exposure. These studies highlight the vital importance of glutathione availability in the alveolar space for AM immune functions and the neonatal lung's defense against bacterial infection [92].

Studies in the adult of chronic ethanol ingestion highlight the role of the glutathione redox state and oxidative stress in AM expression of TGF- $\beta$ , GM-CSF/PU.1 signaling, PPAR $\gamma$  expression, immune functions, and risk of experimental pneumonia [89, 95–98]. In addition to GM-CSF, expression and activity of PU.1 are also dependent on Nrf2 and the antioxidant response element which are downregulated by alcohol-induced oxidant stress [99]. Alcohol exposure also upregulates KLF4 in AMs and treatments with GM-CSF or TGF- $\beta$  enhance or dampen KLF4 expression and binding, respectively [100]. Treatment with siRNA against KLF4 normalizes the effects on expression of GM-CSF and TGF- $\beta$  as well as AM immune functions. A potential dynamic interactive role for chronic oxidant stress on TGF- $\beta$ , GM-CSF, PU.1, Nrf2, and KLF4 on the newborn AM phenotype and immune functions remains to be determined.

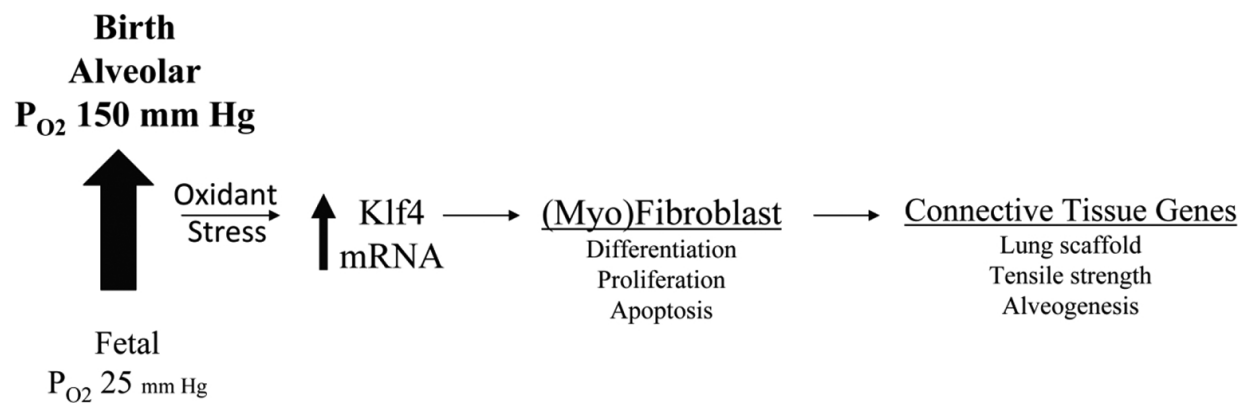
The most common form of newborn chronic lung disease, bronchopulmonary dysplasia (BPD), is thought to be caused by oxidative disruption of lung morphogenesis. Following birth,

various risk factors, including pulmonary or systemic infections, high concentrations of oxygen inspiration, and mechanical ventilation, may act synergistically, amplifying the inflammation. At birth, premature newborns have significantly lower glutathione concentrations than term newborns, and the lowest glutathione levels are associated with the infants with the most severe airways problems and required high oxygen support [101–104]. In intubated newborns, exhaled breath condensate samples correlated with tracheal aspirate samples in terms of reduced, oxidized, and total glutathione (GSH + GSSG) [105]. Decreased glutathione and subsequent oxidative stress in this microenvironment of the lining fluid was also observed in the AMs obtained from the tracheal aspirate. This glutathione deficiency and oxidative stress associated with the immature lung are likely to promote changes in the AM phenotype and immune functions, thereby increasing the risk of infection. This increased oxidative stress may contribute to the significant increased expression of TGF- $\beta$  in the AMs of premature newborns [106, 107]. Further research is needed to specifically decipher the unique and complex mechanisms underlying AM maturation and pulmonary immune regulation at baseline and in response to oxidative stress. Pulmonary immune regulation in the premature neonate may be similar to the adult or it may be dramatically different because of the immaturity of the immune system.

5. Is birth an integrator that balances oxidant stress and antioxidant defense?

We have presented our work and ideas about the lung at birth and its experience of a major change in the oxygen environment. Clearly antioxidant gene induction is part of the developmental program in the late fetal lung and provides protection against oxidant stress. Other antioxidant genes are induced at birth in response to oxidant stress as an added source of protection. These activities are essential to a cell like the alveolar macrophage which must now populate the gas-filled alveolar space, protect against inhaled pathogens, and regulate

Birth Shock Hypothesis



surfactant homeostasis. Nonetheless, the normal lung does not buffer all the oxidant stress it encounters at birth. Rather a transient but measurable degree of oxidant stress persists and we propose that this level serves a physiological function in our **birth shock hypothesis**. As the local redox state of lung cells changes, this signals the lung to initiate an alternative course for postnatal lung development. It is in effect a “**natural experiment with hyperoxia**” as the lung cannot predict the magnitude of the change in the oxygen environment that will ensue. It survives by balancing a level of redox stress that limits lung injury, while permitting activation of genes required for postnatal lung development. Birth then may serve as an integrator of these two competing ends. Our observations about the transcription factor Klf4 (**a birth shock protein**), fibroblast and myofibroblast and macrophage cell populations and the transcriptional regulators we described affecting cell proliferation, cell differentiation, and cell apoptosis in our studies are likely only the beginning of understanding this hypothesis. We believe that study of an event such as this in the newborn lung may provide new insight and strategies to assess, support, and manipulate the oxygen environment and oxidant stress to enable a more normal development for the premature lung at birth and repair of oxidant-induced injury in the developing and the mature lung.

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