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LUNG GROWTH AND DEVELOPMENT

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1. ABSTRACT

The organogenesis of lung involves several complex mechanisms, including interactions between cells originating from two germ layers – endoderm and mesoderm. Regulation of lung branching morphogenesis with reference to its architecture, growth pattern, differentiation, interactions between epithelium and mesenchyme and / or endothelium, as well as genes regulating these processes have been addressed by the pulmonary biologists through careful molecular biology and genetic experimental approaches. The mammalian lung develops by outpouching from the foregut endoderm as two lung buds into the surrounding splanchnic mesenchyme. Several different regions of the foregut are specified to develop into different thoracic and visceral organs. The lung-buds further elongate and branch, and the foregut longitudinally gets separated into esophagus and trachea. In rodents (mice and rats), this occurs around embryonic day 11, where the right lung bud develops into four different lobes and left lung develops as a single lobe. In humans, these processes occur by 3-4 weeks of embryonic development, where the right lung is a trilobar lung and the left lung is a bilobar lung. Several generations of dichotomous branching occur during embryonic development, followed by secularization and alveolarization pre- and post-natally, which transform a fluid-filled lung into an air-breathing lung able to sustain the newborn. During these different developmental stages from embryonic to newborn stage, the lung architecture undergoes profound changes, which are marked by a series of programmed events regulated by master genes (e.g., homeobox genes), nuclear transcription factors, hormones, growth factors and other factors. These programmed events can be altered by undesirable exposure to overdoses of hormones / vitamins / growth factors, synthetic drugs, environmental toxins, radiation and other agents. In the recent years molecular techniques have opened avenues to study specific functions of genes or their products (proteins) *in vivo* or *in vitro* at a cellular or an organelle level, some of these include targeted disruption, knock-in / knock-out genes, *in vitro* mutagenesis, use of sense and anti-sense oligonucleotides. Some of these aspects with reference to regulation of normal lung development and growth and a specific example of pulmonary hypoplasia as an abnormal lung formation are discussed in this review.

2. INTRODUCTION

The developing lung undergoes a series of complex changes during embryogenesis and after birth, which involve structural organization and functional maturity to prepare for extrauterine air-breathing life into adulthood. Lung transforms from a set of out-pouches from the foregut to a fluid filled lung with complex development of airway branches and distal lung differentiation to an air-breathing, gas-exchanging organ critical to life.

Histologic changes in airway branching and pulmonary vascular development become recognizable between different stages of embryonic development, newborn and adult. Rodent and human lungs continue to develop after birth. There are various hormones, growth factors and other agents that regulate different aspects of lung growth and development. Environmental toxins, pharmacological drugs, excess hormones or vitamins and other teratogens can alter the normal developmental processes of lung causing abnormal / defective / hypoplastic lung formation. Understanding of pre- and post-natal lung growth and development provide insights into lung function under normal and diseased conditions, including developmental anomalies.

Pulmonary vascular development is critical for proper gas-exchange in the distal lung structures called alveoli of the air-breathing lungs and also provides nutritional and metabolic needs to the lung parenchyma / distal structures of lung. Pulmonary gas-exchange can be viewed as occurring solely under the control of and within the structures that comprise the respiratory system, whereas O_2 and CO_2 transport is a multi-system process that involves respiratory as well as circulatory systems. The demands of the body for uptake of O_2 and elimination of CO_2 vary considerably in a person's daily life, where O_2 consumption may be at basal levels during sleep and may be several folds higher during a strenuous exercise (details of which are not discussed in this review).

Lung is a metabolic organ as well as an endocrine / paracrine / autocrine organ for complex biological functions requiring physiological homeostasis, which involves 40 or more different cell types. Lung is the only inner organ of the body that is exposed directly and constantly to the external environment through inhaled air during breathing. Thus, getting exposed to infectious microorganisms and environmental toxins. Pulmonary defense mechanisms involve filtration of air in the nasal passage to airway cell ciliary movements, biochemical defenses involving surfactant proteins (-A and -D) and surfactant lipid lining, macrophages, oxidant inhibitors and other immunologic mechanisms. Lung injury, chemical, mechanical or other, raises a question of repair and the mechanisms involved therein. Embryonic fetal lungs heal with ease, whereas adult lungs do not. This draws attention to developing a better understanding of repair / regeneration processes in embryonic / fetal lungs, which eventually may enable development of techniques to induce repair and regeneration in adult lungs.

Extensive studies are conducted in studying effects of hormones such as corticosteroids (dexamethasone), thyroid hormone and retinoic acid (RA). Hormones and growth factors have been used at different doses at different times in development to study their specific effects on different aspects of lung growth and development. Despite positive effects of dexamethasone in stimulating surfactant lipids and proteins, it inhibits septation and therefore the alveolar formation, whereas at specific doses RA can induce septation and therefore alveolarization. Molecular techniques have advanced the field of pulmonary biology to new horizons. Transgenic mice with overexpressing genes or knockout genes have been designed for surfactant proteins, membrane receptors, nuclear receptors, transcription factors crucial in regulation of lung growth and development. These studies have been critical in understanding some aspects of lung branching morphogenesis (growth) and differentiation (development).

3. LUNG FORMATION

3.1. Embryology

The onset of pulmonary development takes place at 3-4 weeks of development in humans, and in rats and mice the pulmonary primordium appears as protrusions / out-pouches of the foregut on day 11 of gestation (1-3). The embryological development of lung or the respiratory system has five different stages of development: in mouse, embryonic lung primordium, days 8-9; pseudoglandular lung days 10-16; canalicular lung, days 16-18; saccular lung, days 18-20; and alveolar stage begins at birth and extends almost 4 weeks into the postnatal period. Saccular phase is extended up to 5 days after birth. So, there is a little overlap between saccular and alveolar stages; and in human embryos the corresponding weeks are 3-4 weeks for lung primordium formation; 5-17 weeks, the pseudoglandular stage during which organogenesis occurs giving rise to the major airways, bronchial tree formation and formation of acinar structures; 16-26 weeks, the canalicular stage during which the peripheral lung development and differentiation occurs along with the pulmonary vascular network development and surfactant synthesis; 24-38 weeks is the saccular stage, where the expansion of the air-spaces occurs and between 36 weeks to 1 or 2 years after birth alveolar formation by formation of secondary and tertiary septa occurs. Beyond these years remodeling of the interalveolar septation and remodeling of the alveolar bed and microvascular maturation occurs.

Initial appearance of the pulmonary primordium is as an endodermally derived protrusion of the foregut, which then proliferates into two lung buds into the surrounding mesoderm (3). Each lung bud develops into left and right lungs, respectively by undergoing branching morphogenesis (figure 1), which is a very complex process regulated by various transcription factors, growth factors, and hormones. Four stages of lung development have been defined by Thurlbeck (4): *embryogenesis,* the development of the embryo and lung primordium; *morphogenesis,* development of lung shape and gas exchanging regions; *differentiation,* development of specialized cell from

Figure 1. Murine lung development: formation of lung bud to differentiated lung with airway branching morphogenesis in neonates.

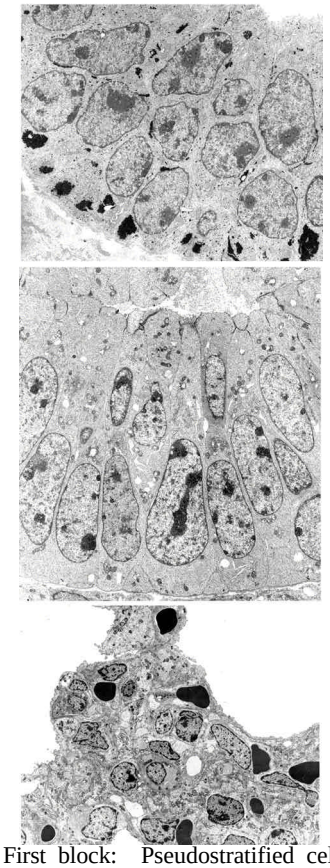

Figure 2. First block: Pseudostratified cells lining the acini of the pseudoglandular lungs. Second block: Tall columnar cells in glandular and canalicular lungs. Third block: Saccular lung with development of future alveolar spaces and extensive capillary network.

precursor cells; and *growth*, enlargement of the lung by volume expansion.

Ten Have-Opbroek and colleagues have described the rodent and human lung developmental stages in extensive details (1, 5, 6). In rodents, the branching tubules of the primordial lungs are lined by undifferentiated tall columnar cells through the pseudoglandular stage of lung development. By day 16 or early canalicular stage of gestation in rodent, the primordial lungs begin differentiating into prospective bronchial structures and prospective respiratory structures, commonly called pulmonary acini. The epithelial cells lining the lumen of the acinar structures, transform from columnar cells to cuboidal cells in the distal respiratory structures and the bronchial structures have columnar epithelium. This stage is comparable to 10-12 weeks in developing human embryo. The ultrastructural differences in the stages of lung development are shown in figure 2 and 3.

As the lung development progresses with the growth of the embryo, the pulmonary acini undergo profound changes resulting into various ducts, saccules or pouch-like structures, which are lined by type II and type I pneumocytes and extensively supplied by the capillary network.

3.2. Development and Structure

There are four distinct structural stages of lung development, each with a characteristic developmental feature. The pseudoglandular stage – development of bronchial tree; the canalicular stage – development of acini and vascularization; the saccular stage – further differentiation of the acini into saccules, increase in saccules and vascularization, as well as differentiation of the epithelial cells into type I and II pneumocytes; the alveolar stage – increase in number of alveoli and extensive increase in the surface area. In rodents (mice and rats) the process of alveolarization mainly occurs post-natally, whereas in humans it is a pre-natal as well as a post-natal process. These four stages of development in the murine lung are shown in figure 4A-D, and figure 4E-H demonstrates the development of vasculature at the respective stages where the vasculature is immunohistochemically stained by PECAM-1 (proliferating endothelial cell adhesion molecule-1).

As the lung develops further, the airway branching continues along with the pulmonary vascular development. The airway cells differentiate into ciliated cells, goblet cells, basal epithelial cells, all of which are tall columnar cells. As the further branching proceeds, the distal regions are lined with a single layer of tall columnar cells. These structures mark the future acini, which are small tubular structures, are surrounded by the mesenchyme. As the tubular structures develop, thinning of mesenchyme around it occurs and the columnar epithelial cells become short columnar and eventually cuboidal epithelial cells. With thinning of mesenchyme, a loose network of capillaries also develops around the acini. During these stages of development the epithelial cell population in the lung doubles and the cuboidal epithelial cells lining the acini have cytoplasm that is heavily laden with glycogen. Basally, the acini are lined by the basement membrane (BM), with no interstitial collagen or elastin in this part of the lung. The major distinguishing features between the pseudoglandular stage and canalicular stage are the development of the pulmonary vasculature and thinning of the mesenchyme.

Further transition from the canalicular to the saccular phase is marked by development of air sacs or the terminal sacs. The proliferation rate of the epithelial cells drops at this stage and differentiation is predominant. The intracellular glycogen in the cuboidal epithelial cells

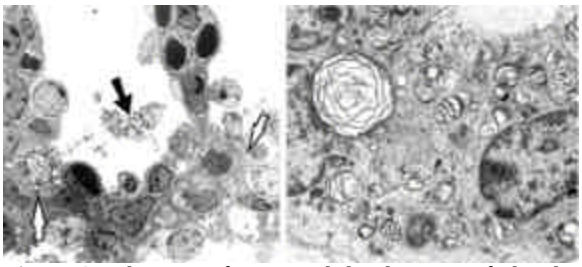

Figure 3. Thinning of septa and development of alveolar structures just prior to and at birth. The arrow shows secretion of surfactant into the alveolar space. The type II pneumocytes are located at the alveolar corners (shown by wide white arrows). The panel on the right shows a few magnified type II pneumocytes with cytoplasmic lamellar bodies.

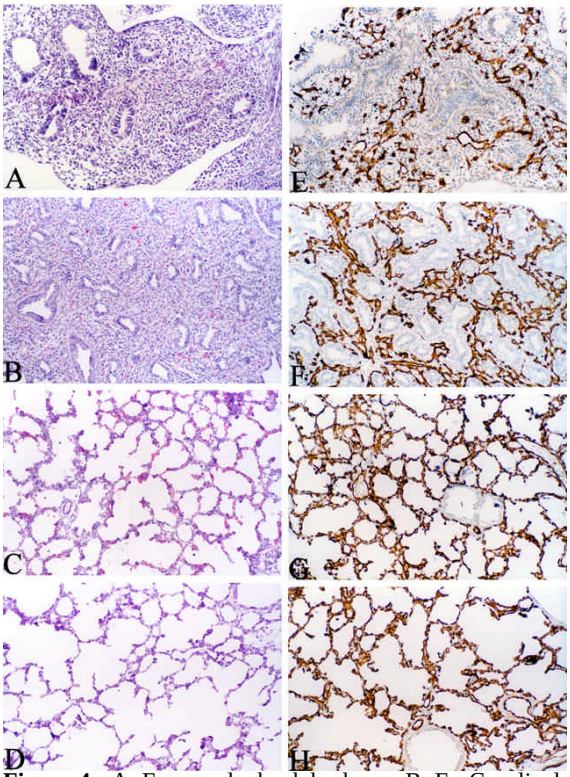

Figure 4. A, E: pseudoglandular lung. B, F: Canalicular lung. C, G: Saccular lung. D, H: alveolar lung. Figures 4A to 4D depict the histological characteristics of four different developmental stages of murine lung (hematoxylin and eosin staining). Figures 4E to 4H show pulmonary vascular network formation in the four steps of developing lung. Pulmonary vasculature is stained by PECAM-1 (brown staining).

reduces dramatically and the formation of lamellar bodies begins, followed by their secretion. From this stage on, the markers of alveolar epithelial cells such as surfactant proteins, several membrane associated proteins, and cytokeratins can be distinctly detected. Gradually, the thinning of the mesenchyme and differentiation of the epithelial cells into the cuboidal epithelial cells followed by further differentiation into type I cells in addition to the type II cells occurs in air-breathing lungs at birth, an alveolar stage of lung. At this stage there are no true alveoli, however the type II cells secrete surfactant.

Initially, the parenchymal or inter-air space septa are broad and have two distinct but interconnected capillary networks. A cellular interstitium is present with low content of collagen and elastin. Prior to the formation of the alveoli, the interstitial cells form elastic fibers, which leads to formation of primary and secondary septa. Along with the expansion of the pulmonary air-breathing surface, the pulmonary vascular branching morphogenesis also increases. The gas exchange surfaces develop as thin linings of epithelial-endothelial cells, where these two cell types share the same basement membrane. Pulmonary microvascular development and its maturation in the lung along with the intra-alveolar septal development is the post-natal process that continues for a few years after birth. The physical forces and the exact molecular mechanisms involved in these structural changes of the different developmental stages of lung remain to be fully understood.

It is an interesting phenomenon, that despite the initial symmetry during the outpouching of the lung buds, the lungs develop asymmetrically on the left and right sides. Several regulatory genes, such as forkhead homologue of hepatocyte nuclear factor (HFH-4), lefty-1 and -2, nodal, Pitx2 (a homeobox gene) are involved in the left-right asymmetry of the lung (7-11).

The morphogenesis of lungs involves several regulatory genes at each stage of development and differentiation and their complex interactions. These interactions involve mesenchymal genes regulating epithelium and vice versa and also endothelial-epithelial gene interactions, which are critical driving forces in the pulmonary vascular development / airway branching morphogenesis. Complex temporal and spatial interactions of several hormones and growth factors are involved in development of lung. Hormones such as glucocorticoids, thyroid hormone, retinoic acid and others have been shown to regulate several of the mesenchymal-epithelial-endothelial interactions during lung development. Significance of some of these is discussed in the following sections.

3.3. Signal Transduction Pathways

To date 17 intracellular signal transduction pathways are known with reference to the identity of their ligands, transduction intermediates, kinases, and targets. These pathways are classified in three major categories: early developmental signal transduction pathways, middle and later developmental pathways of organogenesis, differentiation and renewal, and those used in physiologic functions once the differentiation has occurred. Early developmental pathways include the transforming growth factor-beta receptor pathway, hedge-hog pathway, Notch-delta pathway, Wingless-Int pathway and cytokine receptor pathways. Apoptotic pathways, nuclear receptor pathways and interleukin pathways are organogenesis pathways. Some of the pathways involved in the physiologic functions

are G-protein coupled receptor pathways, nitric oxide receptor pathways, integrin pathway, gap junction pathway and some others.

Early developmental factors regulate the commitment of specific regions of foregut into development of specific organs. Some of the well-established early developmental transcription factors are hepatocyte nuclear factor (HNF)-3beta and thyroid transcription factor-1 (TTF-1). In addition to these transcription factors, the role of Homeobox (Hox) genes, and their modulation during different developmental stages by endogenous retinoic acid gradient have been shown to be of significance in the embryonic development and organogenesis.

3.3.1. Retinoic acid (RA) and Nuclear Receptor Superfamily

It is known that the products of regulatory genes (transcription factors or regulatory proteins) direct the normal development of the lung and exert an influence on specific developmental genes. These regulatory genes include early oncogenes and mesenchymal as well as nuclear transcription factors. Recently, attention has been focused on steroid-thyroid-retinoid nuclear receptor family (glucocorticoid receptor-GR, thyroid hormone receptor-TR and retinoic acid receptor-RAR) with reference to lung proliferation and differentiation during developmental stages and thus affecting lung structure and function. Modulation of one of these receptors can transactivate the other members of the family, which may eventually result in affecting multiple downstream pathways (12, 13).

GR is an ubiquitously expressed transcription factor involved in the regulation of many physiological processes. It is developmentally up-regulated, which coincides with the appearance of the surfactant proteins. GR may play a role in regulation of surfactant proteins and lipids and is essential for preparing the lung for the onset of breathing air at birth (12). To define the role of GR in developing lungs, several mutations have been created in mice. Elegant experiments with targeted disruption of GR have revealed that lung maturation was delayed in mice with low or no expression of GR (14). Furthermore, it was demonstrated that mice with a disrupted GR gene died shortly after birth due to respiratory failure, which indicated an important role of this receptor in lung function (15) and that the lung is an important glucocorticoid target tissue.

Labbe *et al*. (16) measured GR concentration in human lungs at different stages of fetal-growth and after birth. They reported that the lowest concentrations of GR were seen in pulmonary hypoplasia. Chinoy *et al.* (12) validated this observation in the murine model of pulmonary hypoplasia and congenital diaphragmatic hernia. GR-deficient mice have atelectatic lungs at birth without any obvious histologic abnormality (14). Taken together, the mice with targeted disruption of GR (15) and GR deficient human hypoplastic lungs (16) as well as murine hypoplastic lungs (12) have impaired functions.

Thyroid hormone, 3,3',5-triiodo-L-thryomine (T_3), is essential for normal growth, differentiation and development. It has widespread functions in development and homeostasis, although the receptor pathway by which this diversity arises is unclear. T_3- binding proteins have been detected in plasma membranes (17), mitochondria (18), cytosol (19) and endoplasmic reticulum (20). T_3 receptors, TR-alpha and -beta, have sub-isoforms and they act in a ligand-dependent or independent manner and act as transcription factors that regulate growth, differentiation, and development. The most accepted hypothesis is that TRs exert their biological effects by forming heterodimers with retinoid-x-receptors (RXRs). Due to the possible transactivation among the members of the family of nuclear receptors, it may influence some of the critical developmental processes (12). Downstream of activated TR:RXR heterodimer are the key nuclear transcription factors thyroid transcription factor-1 (TTF-1 or Nkx2.1) and AP-1, both of which respectively regulate surfactant proteins and early oncogenic proteins. T_3 has also been shown to influence lipid synthesis in lung, therefore the lamellar body formation in the type II cells, indicating significance of these nuclear receptor pathways in lung growth, maturation and function. Double knockout mice for TR-alpha1 / -alpha2 and knockouts of TR-alpha1 / -alpha2 along with TR-beta1 / -beta2 result in neonatal lethality at 4-6 weeks, whereas those of TR-alpha1 alone or TR-beta1 / -beta2 have normal survival (21). TR-alpha plays a significant role in bone development and temperature regulation, whereas TR-beta has been shown to play a critical role in heart and liver gene regulation.

RA is an oxidative metabolite of vitamin A and is involved in the control of many biological processes including embryonic development. Deficiency or excess of RA has been found to be teratogenic, suggesting the specificity of the requirement of RA during growth and embryogenesis. RA mediates its effects via two types of nuclear receptors: retinoic acid receptors (RARs) and retinoid x receptors (RXRs). These receptors exhibit temporal and spatial expression during development. All-*trans*-RA acts via RARs, whereas 9-*cis*-RA exerts its effects via both RARs and RXRs. Specific expressions of these receptors during development, with specific reference to developing lung as well as levels of endogenous all-*trans*-RA, critically affect various aspects of lung morphogenesis.

RARs have three major isoforms– RAR-alpha, -beta, -gamma, each with multiple sub-isoforms under each category. RARs exist with extensive redundancy, and the deficiency of one of its isoforms can be compensated by another. RARs share a common domain with TR and are known to interact with TR and GR during development. RAR-alpha and -beta have been suggested to play a role in lung morphogenic events such as septation and alveolarization. Both these receptor proteins were reduced in the murine model of hypoplastic lungs. This observation is supportive of the morphologic findings in rodents and humans that there are fewer and less mature alveoli in these lungs compared to the normal lungs of equivalent age (12, 22-26). In recent experiments, Massaro *et al.* (27) have demonstrated that RAR-beta is an endogenous inhibitor of septation. This observation is consistent with the

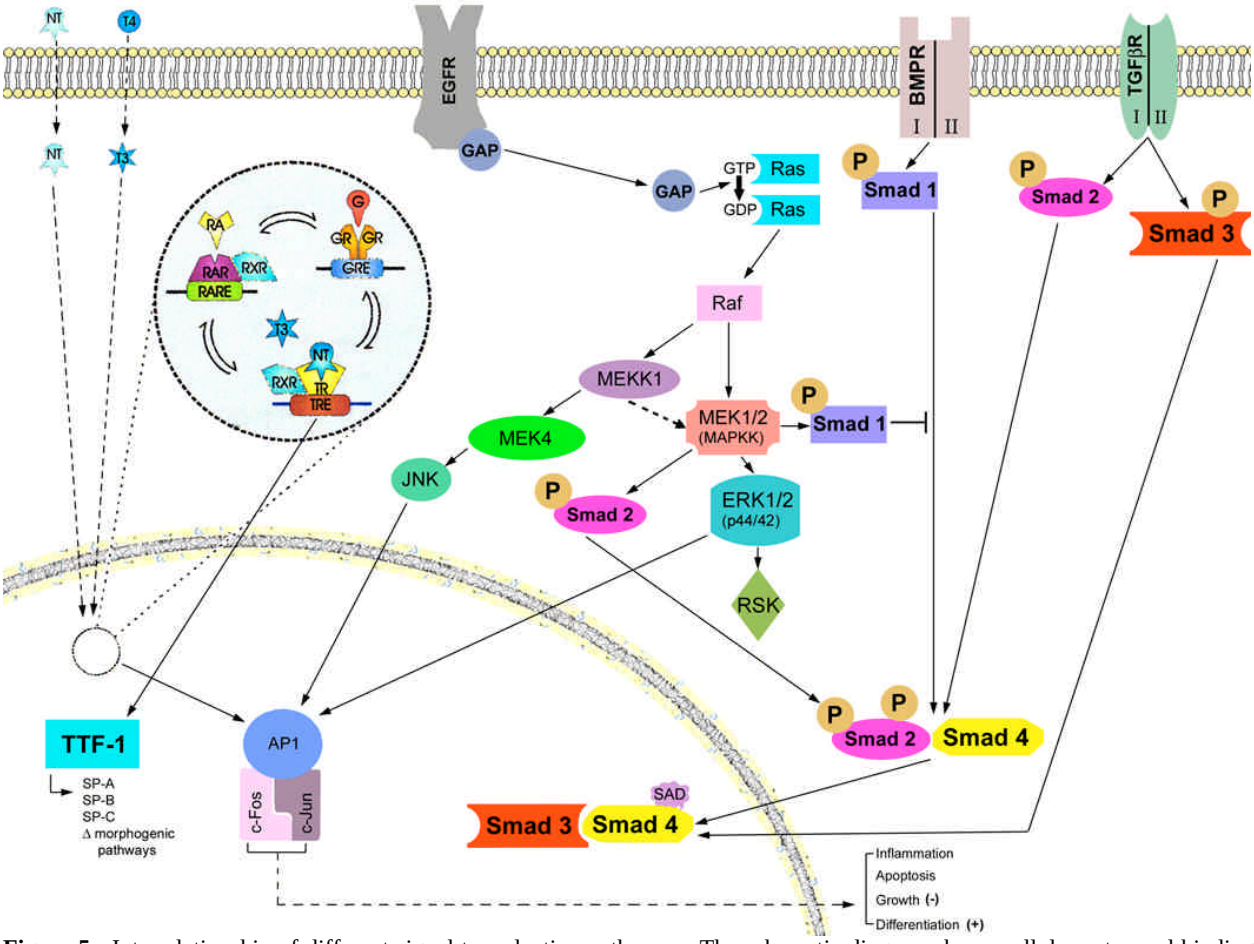

Figure 5. Interrelationship of different signal transduction pathways. The schematic diagram shows cellular entry and binding of T_3 or nitrofen to steroid-thyroid retinoid receptors and the possible downstream molecules that they may affect, as well as the possible interaction between pathways downstream of EGFR, BMPR and TGFbeta receptors.

developmental down-regulation of RAR-beta in lungs. RAR-beta knockout mice exhibit premature septation and form alveoli twice as fast as wild-type mice during the period of septation, but at the same rate thereafter. They also demonstrated that RAR-beta treatment of the newborn rat impairs septation. Their results suggested that molecular signaling regulating alveolar formation differs during and after the period of septation. In addition, it has been demonstrated that lungs of transgenic mice with constitutively activated RAR-beta have immature distal lung structures and collapsed air spaces at birth (28). Furthermore, Hind and Maden (29) have shown that systemic RA given later in life regenerates alveoli in rats with dexamethasone disrupted alveologenesis and Chinoy and co-workers have demonstrated it in the cultured murine fetal lungs and human fetal lung explants that RA induced septation in a dose-dependent manner and dexamethasone inhibits septation (30-32).

Ghyselinck *et al.* (33) demonstrated that RAR-beta mutants are growth-deficient, but are fertile, have normal longevity and appear morphologically normal. RAR-alpha and -beta double mutants display hypoplastic lungs and numerous visceral abnormalities, most of which are incompatible with post-natal life. RAR-beta has apparent functional redundancy with either RAR-alpha or RAR-gamma.

RARs and TTF-1 are known to localize in the bronchiolar epithelial cells of developing lungs and type II cells of the mature lungs (34). TTF-1 is known to interact with other members of the steroid-thyroid-retinoid receptor superfamily, and also regulates surfactant protein B (SP-B) promoter (see figure 5), thus leading to further complexity of the mechanisms involved in lung morphogenesis as well as lung function with special reference to SP-B. Up-regulation of RAR-beta is associated with the terminal saccular stage, with the induction of type II and type I epithelial cells, thus suggesting its role in induction of alveolar development.

3.3.2. TGF-beta Superfamily

Transforming growth factor beta (TGF-beta) family of cytokines is known to regulate cell proliferation, differentiation, recognition, and death. Members of TGF-beta family can be modulated by retinoic acid (RA). Since

TGF-beta family plays a predominant role in the control of development, tissue recycling, and repair (35), modulations of TGF-beta can affect all these critical processes. The TGF-beta family consists of isoforms that have diverged from the bone morphogenic proteins (BMPs). Biochemical and genetic research has shown that TGF-beta family members signal by activating a tetrameric receptor complex of two transmembrane serine/threonine kinases generally known as type I and II receptors, where the TGF-beta binds the receptor II first and then receptor II recruits the receptor I to form the heterotetrameric complex. Both these receptors are required for TGF-beta action in mammalian cells (36).

TGF-beta actions are well defined by their ability to promote extracellular matrix synthesis, inhibition of epithelial cell growth and immunosuppression (37), in various tissues including lungs. TGF-beta1, beta2 and beta3, are expressed at high levels during lung development, however they show distinct spatial and temporal patterns of mRNA distribution. This finding is an interesting feature to consider in designing studies on lung development, because the expression and actions of the TGF-beta isoforms may differ depending on the gestational age (38). The expression patterns of these isoforms strongly suggested a coordinated role of these proteins in mesenchymal-epithelial interactions during embryonic development, as there are marked differences in the patterns of TGF-beta1, -beta2 and -beta3 expression during branching morphogenesis of the lung. *In vitro,* increased levels of TGF-beta1, -beta2 or -beta3 increased accumulation of alpha-smooth muscle actin protein (alpha-SMA) in the mesenchyme and inhibited airway branching, cell proliferation, and expression of surfactant protein C, a marker of distal lung maturity. TGF-beta1, but not -beta2 or –beta3, inhibited branching and proliferation when restricted to the epithelium, but had no effect on alpha-SMA or SP-C, suggesting different signaling mechanisms for -beta1, -beta2 and -beta3 (39). The significance of TGF-beta isoforms in lung is also demonstrated in specific knockout mice of those isoforms, details of which are discussed under transgenic mice – overexpressions and knockouts.

The recent discovery that the TGF-beta superfamily of receptors is involved in the pathogenesis of pulmonary hypertension should lead over the course of the next several years to specific therapies aimed at the origin of the disease. About 50% of the patients with persistent pulmonary hypertension have mutations in exons of the BMPR2 gene or in the area near BMPR2 on chromosome 2, perhaps in a promoter or upstream regulator or perhaps in intronic DNA. About 25% of patients with sporadic primary pulmonary hypertension also have BMPR2 mutations (40, 41). Clusters of endothelial cells carrying somatic TGF-beta2 receptor mutations are found in plexiform lesions in the pulmonary arterioles of patients with sporadic primary pulmonary hypertension (42). The schematic in figure 5 depicts the possible interrelationship and complex mechanisms involving RAR and TGF-beta receptor pathways.

3.3.3. Homeobox (Hox) Genes

The Hox genes are one of the first families of the transcription factors that were described. The Hox genes are expressed in overlapping domains and are responsible for the positioning of major body structures of an organism / individual. Hox genes are a family of transcription factors with helix-turn-helix DNA binding motif (43, 44) that have attracted attention as master regulators of developmental processes. The downstream genes that the Hox proteins regulate are still being defined, so a complete picture of how they work is not yet available. Hox genes are expressed in the developing lung in a pattern that carries the information about position and time, suggesting that they may be involved in the patterning of the lung as they are in the patterning of the extremities, vertebrae, brain, and so on (45).

Hox genes are characteristically organized in four clusters, Hoxa, Hoxb, Hoxc and Hoxd. The genes in these clusters are highly conserved from eukaryotes to mammals. These clusters are located on different chromosomes in mice, chromosome 6, 11, 15 and 2, respectively (46, 47). The genes that are at 3' end of each cluster are early developmental genes and are expressed more anteriorly and the genes that are closer to the 5' end of each cluster are late developmental genes and are expressed more posteriorly. The Hox genes are sensitive to stimulation by retinoic acid (RA) and the activation of the early developmental Hox genes require higher gradients of RA than the late developmental Hox genes (48). In clusters a and b several anteriorly expressing genes have been shown to be expressed in lung (46).

In mouse, the Hoxb cluster, which is found on chromosome 11, has nine genes, and the Hoxb5 gene is located in the middle. Hoxb5 was one of the first vertebrate genes cloned, and its expression has been localized to many embryonic tissues at more than one axial level. Hoxb5 was also the first vertebrate Hox gene found in mesodermal organs such as the lung, kidney, and gonads (49-51). The lung arises from the foregut in a region where Hoxb3-Hoxb5 are highly expressed, thus suggesting that all three genes are important in the initiation of lung morphogenesis. Aberrant expression of Hox genes has been associated with both morphological abnormalities and oncogenesis (52). Temporal and spatial regulation of some of these genes in the lung has been demonstrated in a manner that is collinear with the gene order on each chromosome (53). Furthermore, Shephard *et al*. (54) and Bogue *et al*. (55) demonstrated by Northern blot analysis of both fetal mouse and rat lungs that expressions of Hox genes a5, b5, b6 and b8 are gestationally regulated and decrease with advancing gestational age. Influence of different hormones (dexamethasone), and growth factors (epidermal growth factor [EGF], transforming growth factor-beta [TGF-beta, independently and in combination]), on Hoxb5 protein levels also demonstrated a direct correlation between the advanced development of lung and down-regulation of Hoxb5 protein (56).

With specific reference to Hoxb cluster, expressions of Hoxb2-Hoxb5 were evaluated in murine lung from embryonic day 9.5 onwards (57). They demonstrated that Hoxb2-Hoxb5 are expressed in the branchial arches and developing foregut such that Hoxb2

was expressed in more rostral regions that develop into the pharynx, whereas Hoxb3, Hoxb4 and Hoxb5 were localized progressively caudally along the foregut axis. In the region of the prospective lung bud, Hoxb2 expression was weak, whereas Hoxb3, Hoxb4 and Hoxb5 expressions were stronger. By embryonic day 10.5 of development, Hoxb3 and Hoxb4 were expressed in proximal and distal rat lung, whereas Hoxb5 was restricted to the distal lung. Furthermore, Volpe *et al*. (58) evaluated the level of Hoxb5 protein in mouse lungs from embryonic day 13.5 to postnatal day 2 and showed that, with advancing gestation, Hoxb5 protein became restricted to subepithelial fibroblasts and adjacent columnar and cuboidal epithelial cells of conducting airways. These results suggest a function for Hox genes in the mesenchyme and possibly mesenchymal (mesodermal origin) - epithelial (endodermal) interactions, which are critical for the differentiation of epithelial cell types in the lung (59, 60).

With reference to other Hox genes, studies on Hoxa5 knockout mice demonstrated its essential role during lung development (61). Other members of the homeodomain transcription family are TTF-1 (Nkx2.1, which itself is regulated by a Hox gene), the POU domain proteins, and the Pax proteins. The understanding of the developmental processes such as cell commitment and the initiation of differentiation are required to understand the regulatory aspects of lung development.

3.3.4. Regulation of Branching Morphogenesis

The lung buds of the endodermal origin undergo extensive branching morphogenesis and alveolarization along with pulmonary vascular development (vasculogenesis and angiogenesis) in order to prepare the lung from a fluid-filled organ to the air-breathing organ. While epithelial-mesenchymal interactions are critical during lung morphogenesis, the secreted factors and transcription factors involved in this complex process are yet to be fully understood.

Interactions between epithelium and mesenchyme have been shown to be critical for the morphogenesis of many different organs (62), including the lungs. The epithelial-mesenchymal interactions that drive lung morphogenesis and epithelial differentiation involve complex interactions and signaling between hormones, growth factors and mesenchyme with specific involvement of the extracellular matrix (ECM) molecules (63, 64). Two distinctly different processes are directing the airway development and the distal lung development. Through grafting experiments, it has been demonstrated that epithelial growth and differentiation are regulated by the mesenchyme during embryonic development (65). These experiments demonstrated that tracheal epithelium can be induced to branch, when cultured on bronchial mesenchymal tissue. Recently, Shannon and colleagues confirmed these observations by recombination experiments of the tracheal mesenchyme grafted in the distal lung regions at embryonic day 13-14 in fetal rat lungs (66). The results demonstrated that the mesenchyme drives the epithelial differentiation in the recombinants and that at this stage of development the entire respiratory epithelium has significant plasticity in eventual phenotype of the developing lungs. Furthermore, it has been demonstrated that the mesenchyme isolated from the distal tips of glandular stage lung can induce tracheal epithelium of same age to branch like the distal lung (67, 68). Under the influence of the distal lung mesenchyme, the tracheal epithelium was reprogrammed to express both morphological and biochemical markers of alveolar type II cell differentiation. These experiments validate that mesenchyme has the ability to program the epithelial differentiation. Understanding the mechanisms of the reprogramming of the tracheal and distal lung epithelium may prove to be useful in identifying the factors responsible for modulation of proximal and distal structures of the lung.

Among the regulators of developing lungs are also the early oncogenes, which play a major role in proliferation, differentiation, apoptosis and branching morphogenesis of the lung. Several endocrine and paracrine factors have been identified as modulators of the developing lungs. These factors include various growth factors such as epidermal growth factor (EGF), transforming growth factor beta (TGF-beta) and other related members of the family such as bone morphogenic proteins (BMPs), keratinocyte growth factor (KGF), fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), platelet derived growth factors (PDGF), interleukins, cytokines and many more. Interactions between these factors and their downstream pathways regulate the intricate aspects of growth and differentiation of the lung developmental program. The roles of some of these factors are discussed in different sections in this review. The question about how the primordial lung cells differentiate into a complex organ with over forty different cell types remains unanswered, despite the extensive research efforts in this direction.

Recent advances in the molecular mechanisms and conservation of the developmental pathways have indicated that the key factors involved in the process of branching morphogenesis of the lung are highly conserved through evolution in *Drosophila*, mouse and human. Various developmental pathways such as hedgehog pathway with the downstream FGF molecule, WNT pathway with the beta-catenin and connexin 43 as downstream effectors, nuclear hormone receptor pathways involving steroid-thyroid-retinoid receptor superfamily and downstream complex growth and differentiation pathways, transforming growth factor-beta (TGF-beta) and bone morphogenic protein (BMP) pathways and several transcription factors such as thyroid transcription factor-1 (TTF-1), GATAs (a family of zinc finger factors) have been shown to be involved in normal / abnormal development of lung (13). Significance of various homeobox (Hox) genes has been discussed with reference to lung development, where Hoxa5 null mutants have been shown to exhibit tracheal occlusion and surfactant protein deficiency. Fibroblast growth factor receptor-2 and -4 compound null mutants include perturbed alveolar myofibroblast differentiation and abrogated alveolarization in the neonates (69). Furthermore, Miettinen and

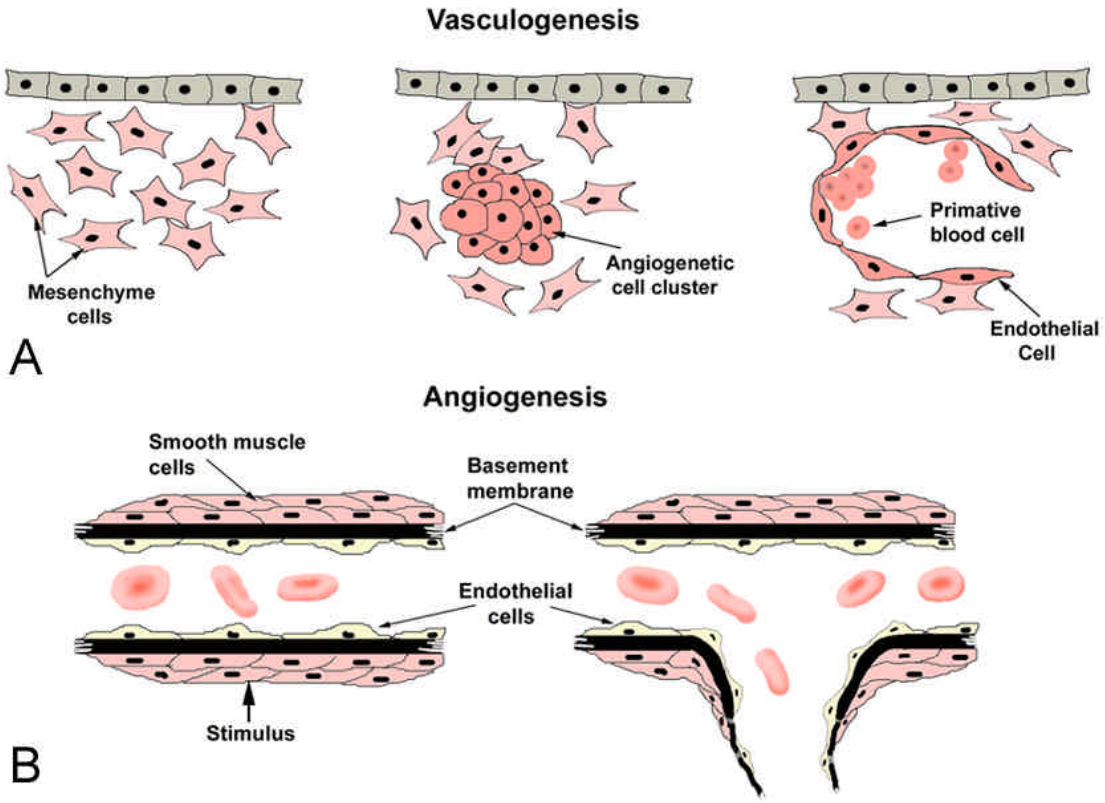

Figure 6. The figure depicts stages of vasculogenesis and angiogenesis.

colleagues (70, 71) demonstrated that the mice deficient in EGFR (-/-) have abnormal airway branching and poor alveolarization, which indicated insufficient maturation of the lungs compared to the lungs of the wild type mice of equivalent age.

Recent studies have indicated that airway branching can be regulated by vascular endothelial growth factor (VEGF), suggesting endothelial-epithelial interactions or pulmonary vasculature driven airway branching morphogenesis. Some of these aspects are discussed in the following section.

4. ANGIOGENESIS AND LUNG DEVELOPMENT

The cardiovascular system is the first organ system to develop in the embryo. Establishment of vasculature requires two distinct processes: "vasculogenesis" and "angiogenesis" (72). Vasculogenesis is defined as the *de novo* formation of the vessels from the mesenchymal precursor cells of endothelium and angiogenesis is defined as sprouting of new vessels from the existing ones (figure 6A and B). As described by Reid and deMello (73, 74), initial vasculogenesis is described as small, apparently empty intracellular spaces in the primitive mesenchyme. These spaces increase in number and size and harbor hematopoietic cells. No connections to the larger vessels exist at this stage, however these vascular lakes eventually connect with the larger vessels by angiogenesis, thus resulting in a vascular network. The pulmonary vasculature develops by both these processes of vasculogenesis and angiogenesis. In the murine lungs at embryonic day nine the intracellular spaces are devoid of the hematopoietic cells, but by day 10 there are numerous spaces with hematopoietic cells, which indicate the establishment of the vascular system.

In humans, angiogenesis is first detected in the developing trachea, esophagus and lung buds at 32 days of gestational age (5^{th} week of embryonic development). Primitive pulmonary arteries become incorporated into the sixth aortic arch, while the intersegmental arteries involute by the end of the 5^{th} week of gestation. Preacinar vessels in the lung, both arteries and veins, develop at the same time as airways. Therefore, the preacinar arteries are present after the 16^{th} week of gestation (75).

Development of pulmonary vasculogenesis is not well understood, despite the fact that the endothelial cells are the most abundant cell type in the differentiated lungs. Mechanisms of vessel formation in the developing lung require the coordination of several processes common to all areas of embryonic cardiovascular development (76). Differentiation of endothelial cell precursors, followed by migration and tube formation, creates a complex, highly organized network of vessels. The complicated scheme of vessel formation has been described as occurring by two mechanisms: angiogenesis and vasculogenesis (72, 77-79).

The structure of the pulmonary arteries varies with vessel size and developmental stage of the lung. Pulmonary vascularization is guided by a well-defined interplay between ECM proteins, cell-adhesion molecules, growth factors and their receptors. The role of growth factors in the developing pulmonary vasculature has been investigated with specific reference to the family of fibroblast growth factor (FGF), transforming growth factor-beta (TGF-beta) and isoforms of platelet derived growth factors (PDGF). Abnormal pulmonary vasculature leads to abnormal development of the lung, which is discussed later in this review under 'abnormal lung development.'

VEGF has two receptors, receptor-1 (fins-like tyrosine kinase [Flt-1] and receptor-2 (fetal liver kinase [Flk]-1, the kinase domain-containing receptor). VEGF and Flk-1 play a significant role in vasculogenesis and angiogenesis, and angiopoetin-1 (Ang-1) is required for vascular maintenance. VEGF expression pattern in developing lungs indicates its role in lung morphogenesis coinciding with vasculogenesis during early development. VEGF protein is developmentally down-regulated in normal murine lungs, which may correlate with the shift in developmental activity toward saccularization and surfactant production in preparation of birth (80). High expression of VEGF results in high permeability of the vascular endothelial cells (81). Ang-1 protein is associated with active recruiting and maintenance of association of periendothelial support cells (pericytes, smooth muscle cells [SMC], myocardiocytes) in an effort to solidify and stabilize the newly formed vessels (i.e., to maintain vessel integrity and quiescence) during this active stage of vascular development. Hanahan (82) suggested that Ang-1 action is mediated via Tie-2 receptors, which activate the pathway of maturation of endothelial tubes into elaborate vessel structures involving the above-mentioned multiple cell types.

4.1. Epithelial-Endothelial Interactions

Mammalian lung development occurs *in vivo* as a coordinated developmental process that includes (1) airway and acinar development; (2) cellular differentiation; (3) biochemical maturation; (4) interstitial development including vasculature and extracellular matrix; and (5) physical growth and enlargement. These parallel processes occur in a coordinated fashion such that at any given time during development there are characteristic relationships among each component that define the stages of lung development.

The developing vasculature, both arteries and veins, follows the development of the airways up to the development of acinar structures and the intra-acinar vessels follow the development of the alveoli (73). These developmental processes result in a structurally and biochemically mature lung, which is well-equipped for air-breathing. Abnormalities in these developmentally coordinated processes may result in abnormal / inadequate lung formation, e.g., hypoplastic lung formation, and may result in respiratory distress at birth.

Proper pulmonary vascular development and pulmonary blood flow are important for normal lung development. Vascular remodeling is important in many biological processes such as wound healing, organogenesis, and growth. Vascular remodeling involves the detection of signals due to hemodynamic conditions and the subsequent delay of those signals to adjacent cells (83). Factors that influence cell growth, death and movement must then be produced, which will affect structural changes in the vessel wall and / or the surrounding interstitial matrix. The endothelial cells, which form the inner lining of the vasculature, are the principal cells involved in these processes. Altered flow or absence of flow is detected by the endothelial cells as shear-stress. The shear-stress may activate the transcription of genes for factors involved in the remodeling of the vessels such as nitric oxide synthase (NOS), platelet derived growth factor (PDGF), TGF-beta 1 and several others. During fetal development, blood flow is critically important to the development of the lung, implicating that the pulmonary blood flow is critical to the development of the pulmonary vasculature.

In abnormal or insufficient lungs, both the vasculature and the airway development are affected. One such commonly studied condition is pulmonary hypoplasia and congenital diaphragmatic hernia (CDH), where the lungs have significant reduction in the number of airways, reduced number of vessels per unit area of lung and also more muscularized arteries (84). In fetal lambs *in vivo* and also in *in vitro* cultured murine lungs, it has been demonstrated that tracheal ligation can reverse the pulmonary hypoplasia (85, 86). Furthermore, DiFiore and colleagues showed that the pulmonary vasculature improves with tracheal ligation and reverses the increase in arterial muscularization in the hypoplastic lungs. These data provide evidence for the inter-relationship of the airways and pulmonary vasculature. It is logical to assume that there is communication between developing airways and developing pulmonary vasculature, however, it is not proven whether branching airways drive the development of vasculature or the vascular development drives airway branching morphogenesis, i.e., epithelial development drives endothelium or vice-versa. Epithelium and endothelium, both have been shown to be regulated by the mesenchyme and the extracellular matrix factors.

Vascular endothelial growth factor (VEGF) and its receptors Flt-1 and Flk-1 have been implicated in pulmonary vessel formation and regulation of the airway branching morphogenesis (87-89), suggesting endothelium driven epithelial development and differentiation. Furthermore, overexpression of VEGF in lung epithelium has been shown to result in abnormal pulmonary morphogenesis combined with an increase in vascularization surrounding the airways (90), indicating a close relationship of the developing vasculature and with lung development. Recent studies on endothelial monocyte activating peptide (EMAP) II was identified as an important anti-angiogenic factor preventing unorganized vessel growth in fetal lungs (91). In the *in vitro* experiments with EMAP II, they demonstrated that inhibition of fetal lung vasculature alters lung

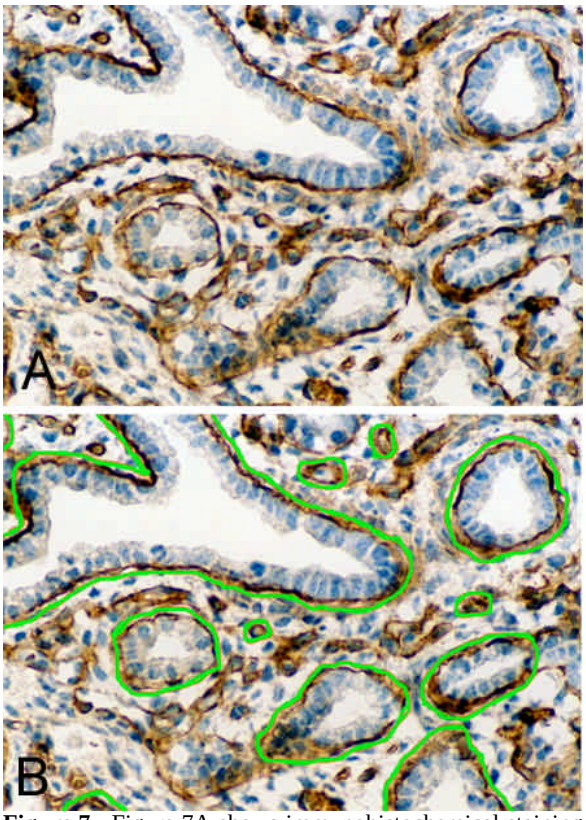

Figure 7. Figure 7A shows immunohistochemical staining for laminin-beta1 in the canalicular lung and the figure 7B shows TN-C staining in pseudocolor superimposed on laminin-beta1, suggesting that TN-C is co-localized with laminin beta-1, however laminin beta-1 is also localized in mesenchymal regions where TN-C does not colocalize.

morphology to an extent that it results in abnormal lung morphogenesis.

4.2. Extracellular Matrix and Basement Membrane

The complex processes of lung organogenesis and maturation involve critical communication between mesenchyme and epithelium. The extracellular matrix (ECM), and in particular the basement membrane (BM), is the medium through which some mesenchymal-epithelial interactions are thought to be mediated and regulated. Several ECM molecules are expressed in the BM. The BM in lung is composed of collagen IV, laminin (figure 7A), heparan sulfate proteoglycan, fibronectin, nidogen, and other proteoglycans (92-94). A complex network of interactions among the cells involve ECM, intermediate filament, and cell surface receptors. Progressive polarization of the cells as a part of the process of proliferation and differentiation involve an ECM molecule, integrin, and various ECM components in the BM or in the subepithelial mesenchyme (95), such as tenacin-C (TN-C) (figure 7B). In addition to the mesenchymal-epithelial interactions, the role of specific ECM components, such as matrix metalloproteinases (MMPs) and their proteolytically cleaved fragments has been shown to govern cell proliferation, differentiation, vascular development and tissue repair. Degradation and reorganization of the ECM plays a significant role in the scaffolding of the vessel walls, where the MMPs, an ever-expanding family of endopeptidases with common functional domains and mechanisms of action are crucial in degradation of ECM components. MMP activity is regulated at multiple levels including the gene transcriptional level and post-translational level.

Cell-ECM interactions involve integrins, which are a large family of heterodimeric transmembrane glycoproteins. They modulate cell adhesion, cell migration, proliferation and epithelial cell differentiation via various signal transduction pathways. The alpha and beta chains of integrins contribute to the binding specificity of each integrin molecule. In mouse embryo, it has been shown that arginine-glycine-aspartate (RGD)-dependent integrins, including alpha 3-beta 1, alpha v-beta 1 and alpha v-beta3, play a role in lung branching morphogenesis (96). The distribution of integrins was recently studied in the very early stages of human lung development (first trimester). The alpha 2, alpha 5, alpha 6, alpha v and beta 1 subunits were detected in plasma membranes of all epithelial cells budding and branching in the mesenchyme (97). Different sets of integrins were expressed upon cell polarization and adhesion.

Another key ECM protein is fibronectin, which is a dimeric glycoprotein. Fibronectin is found in abundance in the embryonic tissue and the protein levels increase during development up to newborn stage, but are reduced in the adults. One of the receptors used by fibronectin to carry out its actions is alpha v-beta 1 (an integrin). Another ECM molecule is TN (figure 7B), which is an oligomeric glycoprotein that is present in the developing mesenchyme. Three TN have been identified so far -C, -X and -R (98). TN-C has been shown to have a morphoregulatory role during development and tissue remodeling. All three TN have fibronectin-like repeats. Antibodies against TN-C have been shown to inhibit airway branching morphogenesis. TN-C positively regulates cell proliferation using distinct cell surface receptors and signal transduction pathways, however a handful of studies have shown that TN-C is able to inhibit cell proliferation (99). Increased levels of TN-C have been shown to inhibit proliferation of vascular smooth muscle cells. It is also suggested that TN-C via influence on basic fibroblast growth factor (bFGF) will lead to aortic endothelial cell sprouting and elongation, therefore down-regulation of TN-C will affect the process. To the contrary of the above observations, the exact role of TN-C remains unclear, as Saga *et al.* (100) demonstrated that mice lacking TN-C develop normally.

Laminin is a non-collagenous glycoprotein in the basement membrane (figure 7A), which is expressed as early as the two-cell stage in mice and RNA levels in the developing lung are widely detected and steadily increase with organogenesis (101). It plays an important role in the development of many solid and glandular organs such as the lung, liver, kidney and salivary gland. Laminin is a heterodimeric molecule composed of three polypeptide chains alpha, beta and gamma linked together by disulfide

bonds. There are about 11 isoforms of laminin known so far, of which laminin-1 (alpha 1, beta 1, gamma 1) is known to be the major isoform expressed in the developing murine lungs. The alpha 1 subunit promotes cell adhesion and has selective mitogenic effects on epithelial cells, thereby stimulating new bud formation. The beta 1 activity appears to be related to branching morphogenesis in the lung, which was demonstrated by using a site-specific monoclonal antibody, suggesting a direct correlation between laminin synthesis and mesenchymal-epithelial tissue formation (102).

Laminin mediates mesenchymal-epithelial interactions, and is responsible for epithelial cell adhesion and polarization (102). Through fetal mesenchymal and epithelial cell co-cultures, it has been demonstrated that cells from early embryonic murine lung can reform organotypically, if cultured on a pure laminin gel or laminin-rich basement membrane extracts (102). Additional studies have demonstrated that laminin is a major mediator of mesenchymal-epithelial interactions (93, 101), thus emphasizing the significance of laminin in lung architecture and development. Thus, various ECM molecules regulate lung morphogenesis through complex interactions among themselves and also with the epithelial as well as endothelial cells in the lung.

5. MARKERS OF DIFFERENTIATION

5.1. Gene Expressions / Transcription Factors Involved

Control of lung growth and morphogenesis with functionally regulated cell-specific expression of genes is a complex process of interactions between several transcription factors and coordinated signal transduction pathways. The differentiated lung has specific alveolar epithelial cell types and well differentiated airway epithelial cells. The alveolar epithelial cell types I and II have been extensively studied. One of the most common cell-specific markers for differentiated alveolar type II cells include surfactant protein-C (SP-C). Among the membrane markers of cell types, *Maclura pomifera* lectin that binds the apical surface of the type II cells is extensively used, whereas *Racinus communis* is a lectin used as a marker for type I cells. The recent literature indicates some of the aquaporins (AQPs) as markers for the different epithelia in the lung. AQPs facilitate water transport across the epithelia. AQP5 is expressed at the apical membrane of columnar cells of the superficial epithelium and submucosal gland acinar cells; AQP4 was detected in basolateral membranes in ciliated ducts and by *in situ* in gland acinar cells; and AQP3 is present in basal cells of both superficial epithelium and gland acinus (103). Furthermore, they demonstrated that the alveolar epithelium has all three AQPs represented, with AQP5 and AQP4 localized to type I pneumocyte and AQP3 to type II cells. Despite their role in osmotically driven lung water transport, AQPs are not required for the physiological clearance of lung water in the neonatal or adult lung (104).

Among the airway epithelial cells the Clara cells are marked by the presence of Clara cell secretory protein (CCSP) (105). CCSP is also commonly known as CC10, which is termed so as it is a 10kDa Clara cell specific protein. In addition to these markers, keratins are also used as specific markers for the different cell types in the lungs.

Significant interest lies in identifying the specific transcription factor for the distal lung modulations, with specific reference to treatments for respiratory distress and induction of lung repair after injury. Some of the most commonly identified transcription factors in the developing lungs are TTF-1 (Nkx2.1), Hox transcription factors, CCAAT / enhancer binding proteins (leucin zipper family; C/EBP alpha, beta, gamma, delta) and hepatocyte nuclear factors (HNFs). TTF-1 and C/EBP have been shown to play a significant role in regulation of the surfactant proteins and both these transcription factors are modulated by hormones such as glucocorticoids and retinoic acid (RA). HNF family is extensively studied because of its tissue-specific expression and regulation of the developmental genes.

6. POST-NATAL DEVELOPMENT OF LUNG

6.1. Architectural Development of Lung

In human newborns the process of alveolarization or formation of the air-breathing surface begins prior to birth in the late gestation stages, whereas in rats this process starts only after birth. Major structural changes in the lung occur at birth, these changes are influenced by the prenatal development of the lung. The prenatal abnormalities may be more pronounced at birth, when the lung actually becomes an air-breathing lung. Lung expansion at birth leads to formation of primary septa and secondary septa, the timing of which correlates with the induction of elastin formation. The air-spaces divided by the primary and secondary septa are called alveoli and the process is called alveolarization. Ultrastructural observations have suggested that the elastic fibers are localized at the tip of the septa that divide different alveolar structures. There are also capillaries localized in these septa. The septa with two capillary layers on either side are considered to be immature septa and as the development progresses they become lined with only one capillary layer and are considered to be mature are-breathing surfaces. Tertiary septation can lead to further generation of alveoli, thus the resulting alveolar clusters.

The process of alveolar formation can be influenced by hormones such as glucocorticoids and retinoic acid (RA) (106-109). In post-natal rats and in fetal murine lungs it has been demonstrated that dexamethasone can inhibit the secondary septation in these lungs and different doses of RA have differential effects on proliferation and differentiation of lungs, where RA (10^{-5}M to 10^{-7}M) have been shown to induce septations (106-109). The inhibition of septation by dexamethasone may be because of the premature induction of the pulmonary microvascular maturation induced by the glucocorticoids (110). Normal lung growth and alveolarization can also be affected by mechanical forces, nutritional status, environmental factors, obesity and various growth factors. Some of which are discussed in this review under other sections.

It has been demonstrated in rat lungs that the secondary septation is accompanied by the active proliferation of cells (111). Further experiments pertaining to DNA synthesis by [^{3}H]-thymidine incorporation assays also demonstrated the thymidine uptake by epithelial type II cells only and not by the type I cells, suggesting the proliferative activities are in type II cells and not in type I cells. These results also indirectly support the fact that the type I cells differentiate from the type II cells (112, 113). Furthermore, the role of type II cells in the adult lung repair processes following an injury has also been established. The type I cells lack most of the cellular organelles and are unable to divide. They have a specialized function of forming the thin air-exchange surfaces.

There is rapid growth of the surface area of the lung by the formation of the type I cells, however these cells do not proliferate. This can be explained by the cytoplasmic extensions of the type I cells and altered morphology of the lung parenchyma. As mentioned earlier, the rearrangement of the alveolar septal surface occurs and the double capillary layers lining the alveolar septa become single capillary layers with expansion of the breathing surface and maturation of the lung. These rearrangements were demonstrated through scanning electron microscopy of the Mercox casts for the rat pulmonary microvasculature Caduff *et al*. (114).

Two major components of the lung, the alveolar surface area and the microvasculature grow in a highly proportionate manner. It is believed that the linear increase in these two components takes place with the growth of an individual during the post-natal period.

7. LUNG INJURY AND REPAIR

7.1. Similarities between Developing Lung and Repair Processes

The natural tissue movements of morphogenesis and the artificially-induced movements of tissue repair in the embryo have been shown to be remarkably close with significant parallels between these two processes (115). Both processes are driven by the changes in cell shape and regulated by the contractions of the actin cytoskeleton. Grose and Martin (115) speculated that the tissue movements drawing a wound closed may, at least partially, recapitulate earlier natural morphogenetic movements. A better understanding of the parallels of wound healing to the developmental processes may be critical in the future treatment of insufficient lungs or injured lungs where induction of repair processes could provide a quality life to the patients.

Grose and Martin (115) asked specific questions for a general comparison of these processes. Addressing the same questions with specific reference to lung may help adult lung repairs in the conditions such as adult respiratory distress syndrome (ARDS) and chronic obstructive pulmonary disease (COPD) and newborn conditions such as bronchopulmonary dysplasia (BPD). The key questions may be: (1) What are the exact kick-start signals that initiate the critical movements? (2) What specific components of the cytoskeletal machinery are required to drive the movements? (3) What are the stop signals for the movement / repair / healing?

It is already established that the wounds in the embryos heal very well in contrast to the adult wounds, which are known for their imperfect healing. A significant role of actin has been shown in the re-epithelialization processes. Furthermore, in the absence of myofibroblasts in the embryonic tissue, the dermal sessile fibroblasts become activated and are recruited to the wound site, where they proliferate and lay down the matrix. This new connective tissue becomes heavily invaded by a network of capillary sprouts from preexisting blood vessels of the wound bed, in one of the rare instances of angiogenesis in adult tissues (116). The other significant differences between the embryonic wound healing and adult wound healing are that the embryonic wounds during re-epithelialization and connective tissue contraction to trigger the characteristic wound angiogenic responses, do not have inflammatory responses, unlike adult tissue. Furthermore, the degranulating platelets and invading macrophages are both largely absent during healing of the wounds in early-stage embryos.

It has been proposed that AP-1 pathway triggers c-Fos, and downstream of this pathway are multiple other pathways of growth/proliferation, differentiation, apoptosis and inflammation, which involve TGF-beta1. Thus, AP-1 signaling may act as a 'kick-start' mechanism to the wound healing process (see figure 5). There is some evidence to show that the tissue movements of morphogenesis and wound repair in the embryo are not just superficially similar, but share common signaling mechanisms. More studies are required in this direction to identify the mechanisms that may be useful in adult lung repair.

Recent investigations on growth factors in the development of lung fibrosis in patients with acute lung injury have emphasized the importance of the growth factors in remodeling the lung tissue after injury. The acute lung injuries could be from multiple causes, such as sepsis, trauma, aspiration and pneumonia. The studies on growth factors and related pathways may be critical in identifying the markers for characterizing specific lung injuries. The markers may help predict the development of the acute lung injury in the patients at-risk and it may also give new insights into pathogenesis of the human conditions (117).

In recent years, cDNA microarrays have been employed to identify the genes / gene clusters involved in pulmonary fibrosis, asthma, acute lung injury and emphysema. The clustering analysis allows identification of genes in a molecular pathway or in the different pathways that trans-activate each other. These studies have provided both expected and surprising insights into the molecular mechanisms underlying pulmonary fibrosis, emphysema and acute lung injury (118). Candidate genes regulating inflammation, tissue remodeling have been identified (119). Interestingly, most of the known TGF-beta inducible genes were included in these clusters, providing support to the hypothesis that TGF-beta6

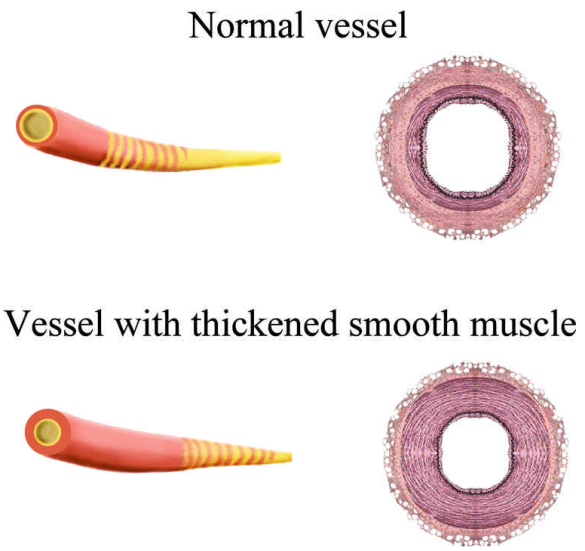

Figure 8. Hematoxylin and eosin stained transverse sections of the normal vessel and that with a thick smooth muscle layer resulting in vasoconstriction are shown in the two panels on the right side.

knockout mice are protected from pulmonary fibrosis as a consequence of failure to activate TGF-beta.

In addition to the significance of TGF-beta in lung injury and repair processes, the fibroblast growth factor (FGF) signaling pathway has been indicated in induction of lung regeneration and repair processes. Furthermore, identification and understanding of the self-renewing population of the progenitor cells or the stem cells in the adult lungs will be helpful in designing novel therapeutic targets to augment lung repair.

8. ABNORMAL LUNG FORMATION

Developmental defects in the lung include (1) vascular abnormalities; (2) tracheal and bronchial abnormalities; (3) hypoplasia or agenesis of one or both the lobes of the lung; and (4) hamartomatous malformations. Normal pulmonary vascular development is critical to normal development of lung. There are four main features by which vascular development may be assessed: (a) branching pattern of the developing vasculature; (b) the number of arteries; (c) wall structure of the developing arteries; and (d) arterial size. Geggel and Reid (120) developed criteria to distinguish between maladaptation, maldevelopment and underdevelopment of the abnormal pulmonary vasculature.

It has been established that the lungs of the human infants with congenital diaphragmatic hernia (CDH) or those of the animal models have significantly reduced numbers of airway and vascular generations (23, 25). Affected newborns have severe respiratory distress because of pulmonary hypoplasia and cardiac abnormalities. Pulmonary arterial abnormalities in CDH human newborns and animal models have been an integral part of hypoplastic lungs (figure 8). Besides the structural abnormalities, functional abnormalities are also seen in the pulmonary vasculature, i.e., vasoconstriction (121). Increased arterial smooth muscle proliferation may contribute to arteriolar vasoconstriction and pulmonary hypertension associated with pulmonary hypoplasia and CDH.

Several extracellular matrix (ECM) components are expressed in the basement membrane during different stages of development and maturation. One such molecule, laminin-beta1, which is one of the key molecules in lung branching morphogenesis, is up-regulated in developmentally advanced lungs compared to less developed lungs. It has been demonstrated that Laminin-beta 1 is down-regulated in hypoplastic lungs, where the lung branching morphogenesis is severely retarded (122). Interactions of mesenchymal molecules as laminin with epithelium may in turn drive the epithelial-endothelial interactions in normal and abnormal lung. It may also drive direct mesenchymal (ECM)-endothelial interactions making it more crucial to understand the signal transduction pathways involved in the developmental processes with specific reference to lung even more complex.

8.1 Pulmonary Hypoplasia (PH) and Congenital Diaphragmatic Hernia (CDH)

Abnormal development of lung can be sporadic or due to genetic inheritance. Formation of hypoplastic lungs and associated congenital diaphragmatic hernia (CDH) in the human newborns has not been found to be occurring due to hereditary abnormalities in the genetic make-up. The recent trend of thoughts is that it is caused in the babies of the mothers with higher susceptibility to the environmental toxins (13). CDH is a birth defect observed in human newborns in which the herniation of abdominal organs is observed in the chest cavity through a hole (incomplete formation) of the diaphragm. This condition is accompanied by cardiopulmonary malfunction, diaphragmatic defect, and depending on the severity of the condition, may also involve gastrointestinal anomalies.

A murine model of a human condition has been created by using nitrofen (2,4,-dichlorophenyl-*p*-nitrophenyl ether), an environmental toxicant (22). Several similarities have been established between this murine model and the human newborn condition of CDH, which include left-sided pulmonary hypoplasia and CDH, reduced airway branching, cardiac and vascular abnormalities, excessive muscularization of pulmonary vessels, surfactant deficiency, respiratory failures at birth, and several other biochemical similarities (12, 22, 86). In this model, it has been shown that the major related abnormality in PH and CDH are cardiovascular abnormalities. The cardiovascular system is one of the first ones to form during organogenesis. Both, an excess and deficiency of vitamin A can result in abnormal heart development. As shown through the studies on the nuclear receptors in the hypoplastic lungs, it was evident that the steroid-thyroid-retinoid receptor superfamily is down-regulated in this condition of PH and CDH (12). Thus, the heart abnormalities in this condition may be attributed to the

abnormalities in RAR pathway and other pathways downstream of it. As shown in the recent studies by Le *et al*., (123) connexin 43 (Cx43), one of the adhesion molecules is involved in the abnormal formation of heart in the CDH mice. In addition, overexpression or underexpression of Cx43 gene has been shown to result in heart malformations (124, 125). Cx43 is a downstream gene to beta-catenin in the WNT pathway (early developmental pathway), thus suggesting the alterations in the WNT pathway. The role of beta-catenin has been proposed in the vascular formation, which further suggests that the modulation of this pathway in the CDH mice results in cardiopulmonary defects, with specific reference to the pulmonary vascular abnormalities.

With reference to the pulmonary vasculature there are abnormalities in the hypoplastic lungs. As discussed earlier, high expression of VEGF results in high permeability of the vascular endothelial cells (81). In murine hypoplastic lungs, the levels of VEGF protein are down-regulated, which suggest that there may be reduced vascular permeability in hypoplastic lungs. It has been demonstrated that nitric oxide (NO) and endothelial nitric oxide synthase (NOS) are also reduced in the hypoplastic lungs (126). NO is important for regulation of smooth muscle cell (SMC) proliferation. These observations suggested that not only NO production is reduced, but its diffusion through the endothelial cells of the vasculature may also be decreased due to the reduced permeability of the endothelial cells in the hypoplastic lungs (80). Thus, reduced production and diffusion of NO may be responsible for excessive proliferation of vascular SMC of the pulmonary vasculature. Based on these data, Chinoy and co-workers suggested that both reduced NO and excessive SMC proliferation are observed in murine model and the human condition affected with CDH and that they may be contributory to the pulmonary hypertension seen in these newborns.

8.2. The Possible Altered Mechanisms of hypoplastic lung formation

Recent cDNA microarray analysis (13) have indicated that expressions of several genes, which are transcriptional regulators with developmental significance, were altered in the hypoplastic lungs compared to the normally developing lungs. Some of these genes were identified as hepatocyte nuclear factors (HNFs), bone morphogenic proteins-2 and -4 (BMPs-2, -4), SMADs, GATAs, MyoD and myogenin, matrix metalloproteinases-2 and -9 (MMPs -2, -9), tissue inhibitors of metalloproteinases (TIMPs), signal transducer and activators of transcription (STATs), epidermal growth factor receptor (EGFR).

Chinoy (13) showed that TGF-beta1 and the related molecules BMP-2 and BMP-4 were down-regulated in the neonatal hypoplastic lungs compared to normal lungs. SMAD3, which is one of the down-stream effector genes of the TGF-beta1 and the BMPs was also down-regulated. These molecules are known to play a role in lung morphogenesis and cytodifferentiation. The difference in the expression of Sonic hedge-hog (Shh) remained to be further explored between the normal and hypoplastic lungs (13).

The GATA transcription factors are down-stream of the BMP-2, -4 and -7 genes. The GATA factors are a group of transcriptional factors that play essential roles in cell differentiation, organ morphogenesis, and tissue-specific gene expression during development. GATA-1, -2, and -3 have significance in the hematopoietic system; whereas, GATA-4, -5, and -6 are nonhematopoietic and are important during the development of embryonic endoderm and mesoderm. We observed down-regulation of GATA-2 gene expression in the hypoplastic lungs. GATA-2 is an endothelial transcription factor. However, the expression of GATA-4 was up-regulated in the hypoplastic lungs. Although, an intrinsic requirement of GATA-4 has been established in heart development (127), the *in vivo* function of this factor needs further attention. GATA-4 knock-out have early-embryonic lethality because of defects in ventral morphogenesis. We speculate that the up-regulation of GATA-4 in hypoplastic lungs compared to the normal lungs may be required to potentiate cardiogenesis and possibly to overcome the defective cardiac formation in the mice affected by nitrofen.

It has been suggested that the endothelial-epithelial interactions may be altered in hypoplastic lungs and the peripheral capillaries were fewer in the hypoplastic lungs due to the defective vascular development (80, 126). A microarray analyses by Chinoy's lab revealed that Connexins 37 and 40, which have endothelial cell specific expression, are down-regulated in the hypoplastic lungs. The connexin gene family encodes the protein subunits of gap junction channels. Gap junctions play a role throughout embryogenesis and are involved in patterning and differentiation. Gap junctions have been suggested in the metabolic cooperation between cells, synchronization of cellular physiological activities, growth control and developmental regulation. Altered expressions of GATA-2 and the endothelial cell specific connexins further suggest that endothelial-epithelial cell interactions are affected in the hypoplastic lungs. It is known that transcriptional regulation of the gap junction protein expression, i.e., connexin expression, can be influenced by ligands of nuclear receptors, such as RA can stimulate Cx43 expression (128) and effects of thyroid hormone on the connexins have been demonstrated through pathological condition of hyperthyroidism involving cardiac problems (129).

The microarray analyses by Chinoy (13, 130) also showed up-regulation of TIMP-4 and down-regulation of MMP-2. Many studies have suggested that extracellular matrix (ECM) proteins TIMPs and MMPs are involved in ECM remodeling and function. Up-regulation of TIMP-4 may be responsible for the proteolytic balance of the vasculature controlling smooth muscle migration and collagen accumulation in the arterial walls of the affected mice. TIMPS are also known to inhibit neovascularization. Based on which they suggested that TIMP-4 may be playing a role in the inhibition of angiogenesis in the hypoplastic lungs and down-regulation of MMPs may

prevent the differentiation of the smooth muscle cells (SMC). Thus, the SMC in hypoplastic lungs proliferate, but may not differentiate well and remain immature. Arterial smooth muscle proliferation has been observed in hypoplastic lungs in humans as well as in the animal models. Furthermore, Chinoy *et al.* (80) showed an up-regulation of the vascular endothelial growth factor (VEGF) precursor in the neonatal hypoplastic lungs, however observed a significant down-regulation of VEGF protein in pseudoglandular hypoplastic lungs. This indicated that the low levels of VEGF protein at a critical stage in development may influence the vasculogenesis in the affected mice. In the neonatal hypoplastic lungs high VEGF precursor mRNA, but low VEGF protein suggests a possible post-transcriptional effect of nitrofen on VEGF.

Other interesting observations by Chinoy and colleagues were up-regulation of inhibitor of MyoD family and down-regulation of MyoD-related proteins and myogenin gene in the hypoplastic lungs. Both MyoD and myogenin belong to the MyoD family of basic helix-loop-helix (bHLH) transcription factors that regulate the skeletal muscle cell differentiation lineage. In myogenin null mice it has been demonstrated that they never inspired air, indicating no breathing movements due to the defects in the skeletal muscle (131). The absence of skeletal muscle and fetal breathing movement *in utero* result in lung hypoplasia in myogenin null mice. These observations suggest the importance of skeletal muscle contractile activity *in utero* for normal lung organogenesis. Our microarray data showing up-regulated MyoD inhibitors and down-regulated MyoD and myogenin suggest that the mechanisms similar to those seen in the myogenin null mice may have also contributed to respiratory distress at birth in the nitrofen-exposed mice due to defects in skeletal muscles and hence, hampered breathing movements.

Signal Transducers and Activators of Transcription (STAT)-3 and -5 are up-regulated in neonatal hypoplastic lungs in comparison to control. STAT signal transduction cascade is initiated when cytokines such as interferons (INFs) or members of interleukin (IL) family or growth factors bind to their cognate receptors. A rapid decrease in STAT3 is reported in differentiating cells and STAT5 is known to appear early after induction of differentiation (132). Therefore, the increase in STAT3 in murine neonatal hypoplastic lungs compared to control lungs suggests delayed differentiation and increased STAT5 probably suggests induction of differentiation in these lungs (13, 130). STATs participate in regulating normal cellular processes, converting stimuli from cytokines and growth factors into appropriate biological responses. Growth factor receptors possess tyrosine kinase activity and phosphorylate STATs (133). A regulatory effect of epidermal growth factor receptor (EGFR) on STAT transcription has been shown (134). It has also been demonstrated that EGFR was down-regulated in the hypoplastic lungs (12) and STAT inhibitor gene was up-regulated (13, 30). Miettinen *et al.* (70) demonstrated that mice deficient in EGFR were growth retarded and they exhibit marked lung abnormalities and breathing problems. Taken together, the data indicated that regulation of development, cell proliferation, differentiation, and apoptosis, in addition to specialized cellular functions, may be altered in hypoplastic lungs due to altered EGFR-STAT pathway. Aberrant STAT signaling can adversely affect these fundamental biological processes by introducing permanent changes in the genetic program.

Based on these results it was suggested that nitrofen-exposure alters some of the early developmental pathways in embryonic mice. The transforming growth factor-beta (TGF-beta) pathway involving BMPs, SMADs etc. and receptor tyrosine kinase pathway involving EGF and the down-stream targets play a role in the abnormal morphogenesis of the lung. Both these pathways may significantly alter the heart, vascular, and lung development. The cytokine receptor pathway involving JAK/STAT is also altered during early development in the nitrofen-exposed embryos. Involvement of early developmental pathways, such as WNT pathway, Sonic hedgehog pathway and Notch-Delta pathway may contribute to the developmental defects seen in the nitrofen-exposed mice. From the organogenesis and cytodifferentiation pathways, there is evidence of the altered nuclear receptor pathway (12, 13, 130). Furthermore, other pathways that are altered in the hypoplastic lungs include the receptor guanylate cyclase (FOS, JUN, AP-1) pathway, nitric oxide receptor pathway, and gap-junction pathway, which are intracellular signaling pathways that play roles once differentiation has occurred.

9. SIGNIFICANCE OF TRANSGENIC MICE: OVEREXPRESSIONS AND KNOCKOUTS IN UNDERSTANDING THE LUNG DEVELOPMENTAL PROCESSES

Transgenic technology has been used to understand gain of function, loss of function via gene targeting (knockout) or loss of function via dominant / negative expression of the gene of interest. Among the various transgenic murine models with specific reference to lung development and function, include the transgenic mice for numerous transcription factors with critical roles at various stages of lung development and differentiation, those for various growth factor receptors and each of the surfactant proteins.

Gene targeting technology has been used to eliminate the genes or knockout (KO) genes to create null mutant mice. The absence of specific gene product helps shed light on the possible developmental function of the specific gene. The KO mice include those for various key transcription factors with a proven role in lung morphogenesis and differentiation, such as KO mice for thyroid transcription factor-1 (TTF-1), hepatocyte nuclear factor 3-beta (HNF3-beta), surfactant proteins (SP)-A, -B, -C and -D, and KO for several nuclear receptors and other transcription factors. The significance of TTF-1 in control of lung-specific surfactant protein genes was demonstrated in TTF-1 KO mice, which lacked the mesenchymal lung structures along with bronchiolar and distal alveolar structures (135). However, these mice had proximal conducting airway structures. The KO mice for HNF3-beta

had multiple germ layers anomalies resulting in absence of foregut development and therefore the absence of its derivatives such as lung development (136). Since, TTF-1 and HNF3-beta are transcription factors involved in early developmental processes, abnormal regulation of these genes result in abnormalities in several other downstream molecules. With specific reference to lung, there are serious resulting abnormalities to its structure and function with loss of function of these transcription factors.

In addition to these null mutants, several others pertaining to the defective lung organogenesis have been created, which are fibroblast growth factor (FGF) 10 (-/-), FGF receptor-3 (FGFR3; -/-), FGFR4 (-/-), sonic hedge hog (-/-), TGF-beta 1 (-/-), TGF-beta 3 (-/-), platelet derived growth factor-A (PDGF-A; -/-), surfactant protein (SP)-A (-/-), SP-B (-/-), SP-C (-/-), SP-D (-/-), retinoic acid receptor (RAR) single and double null mutants. Some of these are discussed in detail in the following paragraphs.

Members of the transforming growth factor beta (TGF-beta) family elicit a diverse range of cellular responses, such as cell proliferation, differentiation, migration, organization and apoptosis (137). TGF-beta isoforms, TGF-beta1, beta2, and beta3, are one of the critical groups of growth factors involved in lung organogenesis. The studies indicate that these isoforms have unique functions and do not have redundant functions in the developmental processes (138-141). Endogenous and exogenous TGF-beta1 has inhibitory effects on lung development and *in vitro* experiments have shown its inhibitory effects on SP-A and -C (138, 140, 142). TGF-beta1 null mutants do not have an abnormal lung phenotype, probably because of the maternal rescue effect (138). Overexpression of constitutively active TGF-beta1 in the distal embryonic lung arrests epithelial cell differentiation and lung saccule formation, thus delaying the development of lung (141). On the other hand TGF-beta3 null mutants have abnormal lung development and they die within 24 hours of birth (143, 144). The abnormal lung morphology of the TGF-beta3 deficient (-/-) mice at birth show alveolar hypoplasia, lack of septation, mesenchymal thickening and less type II cells than in the lungs of the wild type (138). Among all the isoforms of TGF-beta the TGF-beta3 transcript is the most modulated transcript by glucocorticoids (145).

The significance of epidermal growth factor receptor deficient mice (EGFR -/-) has been discussed earlier under "regulation of branching morphogenesis." Briefly, the role of EGF in lung development and epithelial cell maturation has been established (70). Miettinen and colleagues further demonstrated that EGFR -/- mice die shortly after birth due to respiratory distress (70, 71). These mice have low expressions of SP-C and TTF-1, and they are deficient in alveolarization and septation. The molecular mechanisms involved in these defects remain unclear.

Surfactant is composed of 90% phospholipids and 10% proteins, by weight. The proteins are comprised of hydrophilic SP-A and SP-D and hydrophobic SP-B and SP-C proteins. Structurally SP-A and SP-D are very similar and bear resemblance to the mannose-binding proteins (146). SP-A and SP-D have been proposed in the host defense function of the lung. Resting and exercising SP-A-deficient mice (SP-A -/) and resting mice with overexpression of rat SP-A possess normal pools of surfactant, indicating that intra-alveolar surfactant components are not regulated by SP-A (147-151). SP-A deficiency does not compromise lung function in oxygen-exposed animals, but the most compelling phenotypic characteristic of the SP-A -/- mice is a defect in pulmonary host defense.

Unlike the SP-A deficient mice with no phenotypic manifestations of the lung, SP-D KO (-/-) mice had a unique phenotype with decreased SP-A, SP-C and catabolized the endogenous saturated phosphatidyl choline (PC) more slowly than the wild-type mice (152). These mice progressively develop alveolar proteinosis and distal air-space dilation and increased levels of tissue and macrophage-derived oxidants, and phospholipids (153). However, the heterozygous mice for SP-D (+/-) had normal surfactant pool sizes. Similarly, in the mice overexpressing SP-D, normal pool sizes for surfactant were also seen and their lung structures were unaffected. The structural similarity between SP-D and SP-A may be contributory to the decreased SP-A levels in the SP-D KO mice. The role of SP-A has been established in surfactant metabolism, clearance and recycling. This provides an explanation for the findings in SP-D KO with reference to the multiple abnormalities in surfactant components and metabolism.

SP-B knockout (KO; -/-) mice have lethal phenotype and die at birth, since SP-B is critical for the normal function of the surfactant (154). The structural integrity of the secreted surfactant is dependent on SP-B. Deficiency of SP-B has been associated with respiratory distress syndrome in premature infants and in adults.

Recent studies by Thomas *et al.* (155) have shown an association of SP-C gene mutation with interstitial pneumonitis and cellular nonspecific interstitial pneumonitis. This provides evidence that the mutations in the SP-C gene cause dominantly inherited familial interstitial pneumonitis in extended sibship. Accumulation of the mutant protein with possible misfoldings or misrouting might be involved in the pathogenesis of the pulmonary disorder or absence of SP-C and proSP-C can contribute to the severity of the lung disease.

Targeted deletion of SP-C gene in transgenic mice has been shown to result in complete absence of proSP-C in type II cells and SP-C in the airspaces in the murine lungs *in vivo* (156). They observed a modest decrease in surfactant function associated with the abnormalities, however neither surfactant lipid nor proteins were perturbed in the absence of SP-C. Despite these findings, in the inbred strains of SP-C knockout (-/-) mice, severe interstitial lung disease was observed.

It is of interest to mention that the significance of retinoic acid (RA) and its receptors (RARs, RXRs) has

been well established in the developing embryos. KO mice for various RARs and RXRs have been created by Chambon and colleagues (157-159). Deletion of a single isoform of RARalpha did not have any significant effect on the developing mice, but the double mutants were seen with multiple malformations (158, 160, 162, 163), which resembled those observed in the rats with vitamin A deficiency (164), which include the deficiencies of respiratory tract, heart, diaphragm, eye and urinogenital system. Several of these are discussed under the section of abnormal lung formation. The work done by Chinoy and colleagues (12, 13, 22, 86) demonstrated downregulation of all three isoforms of RARs. Furthermore, it has been shown that mice lacking all isoforms of RAR-beta develop normally, but are susceptible to the teratogenic effect of all-trans-RA (165, 166). The complex nature of retinoid-involvement in the developmental defects is evident through elegant observations and data by Chambon and colleagues.

It is appropriate to say that the lung developmental process is complexly coordinated throughout the development by early developmental genes and various signal transduction pathways and its repair during the adult life remains to be understood. This review mentions some of the key areas of lung research and provides and insight into those that remain to be explored.

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