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GROWTH FACTORS IN THE FETAL AND NEONATAL LUNG

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1. ABSTRACT

Formation and orderly development of the mammalian lung results from a complex set of cell to cell and cell to matrix interactions following transcriptional regulation during pulmonary organogenesis. Transcriptional control of differentiation genes early on and epithelial-mesenchymal interactions mediated by growth factors later on, resulting in the formation of conducting airways and an extensive alveolar capillary interface, is critical for normal lung development. HNF-3β and TTF-1 are transcription factors that are involved in gene regulation and formation and differentiation of respiratory epithelium. Studies done in early mouse embryonic lung demonstrate that a variety of peptide growth factors and their receptors are expressed early on in lung development. Signalling through the FGFR2 is critical to normal development of the distal epithelium and mesenchyme. The inductive and permissive influences of growth factors on lung development has been demonstrated by gain or loss of function experiments in early embryonic mouse lung organ culture, in transgenic and in null mutant mice. VEGF present in airway epithelial cells is involved in the maturation as well as proliferation of capillary endothelial cells. Epithelial-endothelial interactions during lung development are important in establishing a functional blood gas interface. Epithelial-mesenchymal interactions mediated by growth factors are also important in the restoration of normal alveolar architecture after lung injury. Further understanding of the role of these growth factors and their cellular interactions in bronchopulmonary dysplasia and in tissue repair following lung injury, may lead to development of better therapeutic modalities in treating these disorders.

2. INTRODUCTION

The lung is a complex organ system whose basic physiologic function is to perform gas exchange across a thin blood gas interface. The lung has the largest surface area of any mammalian epithelium and is capable of supporting a systemic oxygen consumption of between 250 ml/min at rest to 5500 ml/min during exercise (1). Epithelial-endothelial interactions during lung development are important in the establishment of a functional blood gas interface. Developmental disorders involving this interface will almost certainly result in inadequate gas exchange and respiratory distress in infants and children (2). To form such a large diffusible interface with the circulation, the lung epithelium must undergo cell proliferation, branching morphogenesis and extensive alveolarization to increase its surface area, as well as cell lineage differentiation. Both lung morphogenesis and lung cell lineage differentiation are determined by well-coordinated epithelial-mesenchymal interactions. These interactions activate and repress transcription factor mediated mechanisms, peptide growth factor signalling, cell cycle control mechanisms and extracellular matrix expression and signalling (3).

3. TRANSCRIPTIONAL CONTROL OF LUNG DEVELOPMENT

Significant progress has been made in identifying molecular determinants of embryonic lung morphogenesis and cell lineage differentiation during the past ten years. Functional homologies between the genes regulating branching morphogenesis in the tracheal system of Drosophilia and genes regulating mammalian lung morphogenesis are providing new insights into the conserved mechanisms of respiratory organogenesis. Studies of airway morphogenesis in Drosophilia demonstrate that morphogenesis involves both positive and negative regulators; signalling genes are conserved through evolution and signalling molecules function through development. (4). Initiation of lung morphogenesis from the floor of the primitive foregut requires coordinated

transcriptional activation and repression, involving hepatocyte nuclear factor-3b (HNF-3b), sonic hedgehog (Shh), patched (Ptc), Gli2 and Gli3 as well as Nkx2.1 (3). Pattern formation in early embryonic anterior foregut involves Nkx2.1 (thyroid transcription factor-1 or TTF-1), which induces and maintains lung morphogenesis and differentiation of lung epithelial cell lineages (4-6). TTF-1 is a common transactive regulator of the distal differentiated lung cell-specific surfactant protein and CC10 genes. Transcriptional control of each gene requires interactions of TTF-1, with additional transactive regulators such as HNF-3β to account for correct temporal-spatial pattern of expression. HNF-3β is expressed in the developing endoderm prior to lung development and knockout mice lacking HNF-3β have multiple anomalies in all germ layers and fail to develop foregut endoderm or its derivatives (7). HNF-3β expression precedes TTF-1 expression in the developing endoderm and HNF-3β regulates TTF-1 expression. Abrogation of TTF-1 expression by antisense oligodeoxynucleotide gene knock down in culture results in complete interruption of branching morphogenesis and dysplasia of embryonic mouse lung epithelium (6). Nkx2.1 null mice have a severe loss of lung morphogenesis in association with absence of thyroid and pituitary (5). The Nkx2.1 null mutants fail to separate the trachea from the esophagus, suggesting that Nkx2.1 is essential for the development of lung at an early stage (3, 6). Deletion of the TTF-1 gene has been reported in a human infant with both thyroid dysfunction and respiratory failure (6). Absence of Nkx2.1 arrests lung morphogenesis and cell lineage determination at an early stage prior to the specification of peripheral lung cell phenotypes (8). Initiation of Nkx2.1 expression may require morphogenetic signalling by the GATA family of zinc transcription factors (especially GATA-6), HNF-3 and Nkx2.1 itself, and the expression may then be maintained by autoregulation (3). Increased expression of TTF-1 alters alveolarization and causes chronic pulmonary inflammation, demonstrating that precise regulation of TTF-1 is critical for homeostasis in the postnatal lung (9). Abnormal expression of TTF-1 may also contribute to the pathophysiology of pulmonary hypoplasia (10). Subsequent early inductive events mediating tracheal and lung branching, rate of cell proliferation and differentiation and lung angiogenesis involve reciprocal mesenchymal-epithelial signalling mediated by fibroblast growth factor (FGF) ligand receptor signalling as well as modulation by other growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), transforming growth factor beta (TGFβ) and extracellular matrix components including laminin (3). Induction and modulation of embryonic lung morphogenesis by transcription factor and peptide growth signalling mechanisms can occur at a number of levels of integration. These include temporospatial and stoichiometric regulation of ligand, cognate receptor and ligand binding proteins, intracellular effector protein expression and function, transcriptional factor activation and suppression of key developmental gene promoters. These inputs are integrated during the normal process of embryonic, fetal and postnatal lung organogenesis to determine organized patterns of cellular proliferation and cell lineage differentiation, which eventually correlate structure with physiologic function. Lung development extends in a coordinated manner from induction of the lung epithelial rudiment, branching morphogenesis in early embryonic life, through the critical transition from fetal life to air breathing, to the completion of alveolarization, which occurs postnatally (3).

4. GROWTH FACTORS AND LUNG DEVELOPMENT

Signalling molecules (cytokines and growth factors) are the critical messages in intercellular communications. Branching morphogenesis and cell lineage differentiation occur spontaneously in E11 early mouse embryonic lung under serumless chemically defined conditions as well as in zero gravity, suggesting that autocrine and paracrine signalling factors produced within the lung are necessary for embryonic phase of lung branching morphogenesis to occur. Signalling factors that have been characterized include hormones, growth factors, interleukins, cytokines, several biologically active lipids and a variety of molecules including inorganic gases, steroids and retinoids (11). Usually signalling molecules are active at very low concentrations, have a very short half-life and their synthesis and expression are almost always under tight biochemical control. The cellular receptors that recognize signalling molecules do so very efficiently and bind them with high affinity. These receptors are very specific for the signalling molecules that they recognize, although in some cases they bind to more than one ligand. For example, the EGF receptor (EGFR) recognizes both EGF and transforming growth factor alpha (TGFα) and a number of other related factors (12). Frequently signalling receptors undergo desensitization, either by internalization or inactivation after binding their ligands. This provides a mechanism for limiting the duration and magnitude of the responses of the target cells to signalling molecules (13).

Studying the role of individual signalling molecules in complex tissues and in complicated biologic responses has required innovative experimental strategies. These include simplified or “reduced” models of complex signalling events, immunolocalization of signalling factors; blocking the actions of signalling molecules with neutralizing antibodies or by competition with pharmacologic or naturally occurring receptor antagonists; determination of the timing of expression of the genes for protein or polypeptide signalling factors and their receptors by *in situ* hybridization and immunocytochemistry, and creation of mutant cells or animals that overexpress or are deficient in signalling molecules or their receptors (11). Studies of cell-cell interactions in development in Drosophila melanogaster and Caenorahbditis elegans invertebrate systems, and Xenopus and the Zebrafish (Danio rerio) vertebrate models, have been particularly informative (13). Cell to cell communication is of paramount importance in the developing lung (4) and the role of the individual signalling molecules and their receptors are being studied in these models. Because events of development are often mimicked or recapitulated in remodeling or damaged organs, similar strategies are

being used in models of injury to identify actions of signalling molecules acting alone or in concert with other factors (14). These models may lead to new strategies for favorably manipulating repair of the injured lung. Although the simplest system involves a message between a signalling cell and the target cell that is transmitted by a single signalling molecule, this is rarely the case in multicellular tissues. It probably also occurs infrequently in single cell organisms. Signalling molecules tend to be generated in combination by signalling cells, and this combinational stimulation is one of the key ways in which diversity of response is induced in target cells. Such combinations of signals are integrated at points of interface between intracellular signal transduction pathways (11).

Growth factors whose signalling peptides and cognate receptors are expressed in early mouse embryonic lung include fibroblast growth factors (FGFs) including keratinocyte growth factor (KGF), EGF, TGFα, insulin-like growth factors (IGFs), platelet derived growth factor (PDGF), hepatocyte growth factor (HGF) and TGFβ (3). Their inductive or suppressive influences on lung development have been demonstrated by gain or loss of function experiments in early embryonic mouse lung organ culture, in transgenic and in full mutant mice (15, 16). In general, peptide growth factor cognate receptors with tyrosine kinase intracellular signalling domains such as the EGFR, FGF Receptors (FGFRs) including the keratinocyte growth factor receptor (KGFR), c-met, insulin growth factor receptor (IGFR), and platelet derived growth factor receptors (PDGFR) stimulate lung morphogenesis, while those cognate receptors with serine-threonine kinase intracellular signalling domains such as TGFβ family of receptors are inhibitory (17-21).

The biologic activity of several growth factors is regulated by cytokine binding proteins. They do not transduce a cytokine signal, as would be the case for a "cytokine receptor". Cytokine binding proteins act as modifiers that alter the meaning of a cytokine signal perceived by the responding cell. A binding protein present in the microenvironment traps the cytokine and alters the growth promoting activity of the cytokine in either a positive or a negative fashion. The binding protein may serve as a mechanism for cytokine clearance and degradation if a specific cytokine-binding protein receptor exists on the cell residing in the immediate vicinity. The actions of cytokine-binding proteins in regulating some of the growth factors have been reviewed (22).

4.1. EGF, TGFa and EGFR

EGF and TGFα are polypeptides that play a role in the regulation of fetal lung development and epithelial repair after injury (17, 23-28). Both are EGF receptor (EGFR) ligands and can positively modulate early mouse embryonic lung branching morphogenesis and epithelial cell differentiation, which is regulated by mesenchymal-epithelial cell communications (17-19, 23, 29-37). Both EGF and TGFα have been localized to respiratory epithelial cells by immunohistochemistry. EGF is detected in the conducting airway epithelial cells of the developing mouse, rat and human lung (17, 23, 30) and in alveolar type II cells, nonciliated cells of bronchioles, smooth muscle and interstitial and alveolar cells of the alveolar septal regions of developing and adult rat lungs (24, 30). EGF receptors are localized in the conducting airway epithelium of fetal ovine lung, suggesting that the airway epithelium is a target site for EGF and TGFα action (31). EGF, TGFα and the EGFR are colocalized in airway epithelium in normal fetal and in postnatal human lung (23). Colocalization of these growth factors with their receptors in developing lung suggests that they may act through an autocrine mechanism (23). The regulation of EGFR in late gestation fetal lung fibroblasts may control the timing of mesenchymal-epithelial cell communication leading to surfactant synthesis. Factors that affect lung maturation such as like EGF, cortisol, TGFβ1, dihydrotestosterone and retinoic acid regulate EGFR in a developmental, sex-specific manner during late gestation (32). EGF and TGFα, may guide lung development in part by inducing the expression of matrix-degrading metalloproteinases (33). Preterm lambs treated with intravenous infusion of EGF 5 days prior to delivery, showed epithelial cell hyperplasia of the conducting airways and no evidence of hyaline membrane disease compared to preterm controls (34). A combination of growth factors in a mutually interactive environment *in vivo* is more important in regulating lung development. The combination of EGF and TGFβ produces a more normal pattern of lung branching and morphogenesis in fetal mouse lung organ cultures (35). Addition of EGF and TGFβ1 accelerates lung development and maturation of nitrofen-exposed hypoplastic murine fetal lungs in organ cultures compared to untreated controls (36).

Stimulation of EGFR signalling with exogenous EGF/TGFα stimulates early murine embryonic lung branching morphogenesis, epithelial and mesenchymal cell proliferation and Nkx2.1 and Surfactant Protein C (SP-C) expression as markers of cytodifferentiation (17). Abrogation of EGFR signalling with tyrphostins (17), EGFR antisense oligodeoxynucleotide gene knockdown (19), EGF deficiency induced by autoantibodies against EGF (29) or EGFR null mutation (18), all result in decreased branching morphogenesis in culture and a neonatal pulmonary lethal phenotype in the null mutant, associated with decreased branching morphogenesis and Nkx2.1 and SP-C expression (17-19, 29). EGF also is expressed in mature alveolar epithelial cells and regulates type II cell proliferation through an apparent autocrine mechanism both in culture and *in vivo* (15). Neonatal EGFR deficient mice often show evidence of lung immaturity, which can result in visible respiratory distress (37). The lungs of EGFR deficient mutant mice have impaired branching and deficient alveolization and septation, resulting in 50% reduction in alveolar volume and, thus, a markedly reduced surface for gas exchange. The EGFR inactivation also results in type II pneumocyte immaturity, which is apparent from their increased glycogen content and a reduced number of lamellar bodies. EGF treatment stimulates the expression of SP-C and TTF-1 in cultured normal lungs, but not in EGFR deficient lungs, suggesting that EGFR signalling regulates the expression of these marker genes during type II cell maturation (37). This data indicates that signal

transduction through the EGFR plays a major role in lung development and that its inactivation leads to respiratory distress syndrome (RDS; surfactant deficiency; hyaline membrane disease). Both EGF and TGFα stimulate type II epithelial cell proliferation (38). TGFα and EGF increased and TGFβ decreased *in vitro* proliferation of isolated rabbit type II alveolar epithelial cells. TGFβ inhibits the mitogenic effect of EGF or TGFα. If TGFα is added before TGFß, the ability of TGFß to block the mitogenic effect of TGFa is diminished the later in time TGFß was added (38).

EGF and TGFα may play a role in cellular responses after lung injury. They are thought to act locally to stimulate cell proliferation and matrix deposition by interstitial lung cells resulting in pulmonary fibrosis. EGF is detected in cells of the conducting airways of infants dying of bronchopulmonary dysplasia (BPD, chronic lung disease of prematurity), but is not detected in the lungs of infants dying from other causes (24). Lipopolysaccaride endotoxin induces the production of TGFα by alveolar macrophages, supporting a potential role for TGFα in response to infection (25). TGFα and EFGR expression by pulmonary epithelial cells increases after bleomycin-induced lung injury 6 (28). There is evidence that endogenous TGFα is increased in bronchoalveolar lavage of neonatal rabbits exposed to hyperoxia, and that this exposure also delays the appearance of the more mature, lower molecular weight isoforms (39). Pulmonary epithelial cell overexpression of TGFα, using the SP-C promoter in transgenic mice, disrupts alveolar morphogenesis and produces pulmonary fibrosis, mediated by paracrine signalling between respiratory epithelial and interstitial cells of the lung (26). EGF increases with increasing gestational age (GA) and increasing postnatal age (27). The prominent expression of these growth factors in alveolar macrophages in BPD suggests they may be involved with the pathogenesis of the disease and it may predispose preterm infants to develop BPD. At birth EGF in bronchoalveolar lavage fluid from infants with BPD and RDS was lower than control infants. EGF increased in all groups with a more rapid increase in control infants (27). Large numbers of alveolar macrophages immunostained for EGF, TGFα and EGFR in lungs with late stages of BPD, suggesting they may be involved in the pathogenesis of the disease (23).

4.2. Fibroblast growth factors (FGF) and their receptors (FGFRS)

FGF signalling, a complex epithelial-mesenchymal interaction, is an essential component of the regulatory network present in embryonic lung controlling proliferation, differentiation and pattern formation (3, 8, 40). In both the developing tracheal system (in fruit fly) and mammalian lung, an FGF signalling pathway is reiteratively used to pattern successive rounds of branching. The initial pattern of signalling appears to be established by early, more global embryonic patterning systems. The FGF pathway is then modified at each stage of branching by genetic feedback controls and other signals to give distinct branching outcomes (3, 8, 41). Emerging homologies between human, mouse and Drosophilia fruit fly FGF signalling genes and the respective gain versus loss of function phenotypes, suggest that activation of FGFR by FGF family peptides and modulation of FGF availability of FGF-binding proteins such as sprouty are conserved throughout evolution (42). Sprouty family genes act as inducible negative regulators of FGF signalling, which in part may determine the inter-branch length during bronchial development. Null mutation of sprouty resulted in a strong gain of functional phenotype in tracheal branching in Drosophilia (8). Knocking down mspry-2 expression, a murine homolog of Drosophilia sprouty, using an antisense oligodeoxynucleotide strategy, results in a significant increase in branching morphogenesis by early murine embryonic lung in culture (3, 42). Thus genes in the FGF signalling pathway are prime candidates for a major physiologic role in mediating the inducting effects of mesenchyme on the primitive respiratory epithelium (8).

A restricted number of FGF family ligands and all known FGF Receptors (FGFRs) are expressed in the embryonic lung and their expression is regulated in time and space (3). Acidic FGF (aFGF, FGF1) binds to all FGF receptors, localizes to airway epithelium (30) and enhances *in vitro* branching morphogenesis (43). Basic FGF (bFGF, FGF2) is localized to developing airway epithelium, basement membrane and extracellular matrix (30). aFGF, KGF (FGF7) and FGF10 are critical mesenchymal factors that mediate proliferation and branching morphogenesis of the developing respiratory epithelium (43, 44). Some aspects of differentiation appear to depend on a specific ligand: in alveolar epithelial type II cell cultures, KGF can induce a type II cell-like phenotype, whereas aFGF cannot, even though KGF and aFGF bind to the same receptor FGFR-2IIIb (KGFR) subtype (43). However, some effects of FGF ligands appear to be determined by temporospatial distribution of FGFRs and heparin sulfate proteoglycans, which also influence ligand-receptor interactions. aFGF induces epithelial budding in early rat embryonic lung cultures at sites with the highest expression of FGFRs. FGF10 is expressed at high levels during lung development in the distal mesenchyme at prospective sites of bud formation (44). In cultured embryonic lungs, FGF10 soaked beads attract distal epithelial buds that eventually surround the bead, suggesting that FGF10 may be acting as a guidance signal for the distal epithelium but not proximal lung epithelium (45). But FGF10 did not interfere with epithelial differentiation and had a weak effect on proliferation.

Alterations in FGF signalling during embryonic organogenesis result in dramatic abnormalities of epithelial branching and cyto-differentiation (3). Expression of a truncated, dominant negative, kinase deficient FGFR2-IIIb mutant, in the primitive respiratory epithelium of transgenic mice under the control of the SP-C promoter results in a lethal phenotype comprising complete pulmonary aplasia distal to the mainstem bronchial branches (46). In these mice, tracheal cell lineages are normally expressed, but peripheral epithelial cell lineages marked by SP-C expression as well as vasculature are not. Thus FGFR2 signalling is clearly essential for distal pulmonary epithelial

branching morphogenesis and differentiation as well as embryonic pulmonary angiogenesis (3). Pulmonary hypoplasia with abrogation of FGFR signal transduction has been confirmed by generalized overexpression of a dominant negative soluble form of the FGFR extracellular domain (47). Double null mutations of FGFR3 and FGFR4 results in a postnatal lethal pulmonary phenotype in which excess elastin is laid down and alveoli fail to form (48). Congenital Diaphragmatic Hernia, which carries a high mortality of 60% because of associated anomalies (pulmonary hypoplasia, pulmonary hypertension and type II cell dysfunction), may represent an abnormality of FGF signal processing. Intrinsic abnormality of FGF processing has been demonstrated in the hypoplastic nitrofen lung before diaphragmatic malformation (49). Null mutation of FGF10 in mice results in complete abrogation of pulmonary morphogenesis distal to the 7th tracheal cartilage as well as absence of limbs, establishing that FGF10 signalling plays a critical role in organizing both lung and limb development (50). The pulmonary phenotype of FGF10 deficient mice is strikingly similar to that of the Drosophilia mutant branchless, an FGF homologue. In contrast, overexpression of KGF driven by the SP-C promoter in transgenic mice results in pulmonary malformation resembling cystic adenomatoid malformation (51). KGF treatment of murine lung cultures results in a similar phenotype (16). Both of these seem to represent an abnormality of fluid secretion (52). Recent studies have shown that stimulation of liquid secretion in fetal lung explants by cAMP may be mediated in part by induction of KGF expression (53). KGF and FGF10 may be important paracrine factors regulating liquid secretion in human fetal lung. Absence of aFGF and FGFR-2IIIb, the obligate common receptor for KGF and FGF10, has been demonstrated in the fistulous tract in tracheo-esophageal fistula (54). This absence of a specific FGF signalling pathway, may represent arrested development resulting in this congenital abnormality.

In embryonic lung epithelium, growth effects of FGFs appear to be dependent on location of FGFRs, while effects on differentiation are ligand-dependent (3, 55) . KGF and aFGF are potent mitogens in stimulating fetal pulmonary epithelial cell proliferation (55, 56). aFGF and KGF induce a distinct pattern of growth and differentiation in embryonic lung epithelium (55). aFGF stimulates epithelial proliferation that results in bud formation (branching), while KGF stimulates epithelial proliferation that leads to formation of cyst-like structures. In addition, KGF stimulates epithelial differentiation, stimulating expression of SP-A and SP-B throughout lung explants and inducing focal areas of highly differentiated cells. The FGF-induced patterns of growth appear to correlate with the distribution of epithelial FGFRs mRNA (55). Differential expression of the various FGF receptors 1 to 4 and ligand genes has been reported in late fetal and early postnatal rat lung (57). In the presence of aFGF, bFGF, and KGF, the primary lung epithelial cells could be propagated for generations and grown for more than two months *in vitro* (58). KGF was the strongest stimulator of cell growth, whereas bFGF was the most effective inducer of lung epithelial cell-specific surfactant protein expression (SP-A, -B and –C) (58). KGF increased mRNA levels for SP-A and SP-B in adult rat type II cells, suggesting its role in maintaining alveolar epithelium (59). KGF-induced phospholipid synthesis in fetal type II cells occurs independent of its mitogenic effect by stimulating the enzymes involved in surfactant synthesis (60). FGF10 is expressed dynamically in the mesenchyme adjacent to the distal epithelial buds, from the earliest stages of lung development (44). The temporal and spatial pattern of gene expression suggests that FGF10 plays a role in directional outgrowth, proper positioning and possibly induction of epithelial buds, and that positive and negative regulators of FGF10 are produced by the endoderm (44). Even though aFGF1, KGF, and FGF10 have overlapping activities *in vitro*, their *in vivo* expression patterns are quite distinct in relation to early branching events. FGF10 appears to be the key regulator in distal epithelial branching and differentiation, laying the framework for postnatal complete alveolization. Activity of FGFs may be regulated by a combination of positive and negative factors including Sonic hedgehog (Shh), other FGFs, bone morphogenetic protein-4 (BMP-4) and TGFβ (40).

FGF signalling is essential for early lung development and maturation, but also may be very important for postnatal modeling and repair of alveoli following lung injury. KGF enhances the maturation of fetal alveolar type II cells (59, 60) and also plays a major role in mediating glucocorticoid-induced fetal lung maturation (60). Tracheal ligation is accompanied by an upregulation of KGF protein and gene expression, even though it is not clear whether KGF is solely responsible for the growth observed in these tracheal ligation preparations (61). Acute hyperoxic lung injury remains a major factor in the development of BPD in preterm neonates. KGF induces type II cell proliferation *in vitro* and *in vivo*, a critical step in lung injury repair (62). KGF may also be involved in lung repair after epithelial cell necrosis following bleomycin-induced lung injury (63). Hyperoxia increases endogenous KGF mRNA expression in neonatal rabbit lung (63). Intratracheal KGF stimulates alveolar epithelial cell proliferation and protects the lung against oxidant lung injury including hyperoxia (64). Rats treated with rhKGF via the Intratracheal route exhibited a dramatically reduced mortality and had minimal hemorrhage and exudates within the intraalveolar spaces in the presence of hyperoxia (64). It is possible that endogenous upregulation of KGF during the acute phase of hyperoxic lung injury enhances epithelial repair and is important in minimizing later BPD. Of great recent interest are the inducible pulmonary epithelial cell KGF and FGF10 overexpressing transgenic mice models (65). These mice should be extremely useful in delineating both the role of KGF during different stages of development and the potential positive and negative effects of KGF in post-injury repair. KGF and FGF10 overexpression also clearly induce a strong inflammatory response (66).

A secreted FGF-binding protein (FGF-BP), specific for FGFs has been described that binds aFGF and bFGF in a noncovalent reversible manner (67). Unlike, most other growth factors and cytokines, FGFs are not

typically exported by cells into their medium and they lack a secretory signal peptide (68). Instead, they are deposited into the pericellular matrix and immobilized there by binding to Heparin sulfate proteoglycans (HSPGs) (69). The secreted FGF-BP appears to solubilize bFGF from its extracellular matrix storage site and allow it to reach and activate its cell-surface signalling receptor (70). a_2-macroglobulin (a_2M, see Section 2.4) appears to serve as a negative regulator of bFGF (71). bFGF forms an irreversible bond with fast a_2M, and this complex has a diminished ability to bind either high or low affinity FGF cell surface binding sites. Thus a_2M appears to function as a clearance mechanism for bFGF. Heparin sulfate proteoglycans (HSPGs) are critical to the biological activity of bFGF and aFGF. bFGF has a strong affinity for heparin (72) and it is bound to HSPGs in extracellular matrix and basement membranes (73). HSPGs potentiate the mitogenic effect of aFGF and bFGF on endothelial cells and this is due to a heparin-induced conformational change of bFGF that is necessary for high-affinity binding of FGF to its cell surface receptor (74). Thus HSPGs at the cell-surface are required for FGF to exert its maximum biologic activity. Extra cellular matrix HSPGs function to protect bFGF against proteolytic degradation (75) and serve as a reservoir for extracellular FGF, where they can be later released by heparinase (73).

4.3. IGF - peptides and their receptors

The IGFs (Insulin Like Growth Factors) and their receptors and binding proteins (IGFBPs) are expressed endogenously in a number of tissues including the lung during fetal and neonatal development and are involved in differentiation of tissues (56). The endogenous autocrine/paracrine IGF system together with endocrine sources contributes to the regulation of lung cell proliferation. The contrasting levels of expression of different components of the IGF system (IGF-I, IGF-II, IGFRs & IGFBPs) in the fetal lung and liver indicate organ-specific regulation (56). The expression of IGF-I, IGF-II and the type I receptor throughout gestation in the lung supports a role for IGFs in lung growth and development (76). IGF-I is localized to mesenchymal cells surrounding the airway epithelium, whereas IGF-II, which is more abundant, localized predominantly to epithelium in fetal rat lungs from 15 to 21 days gestation. The complex pattern of IGFBP expression (differing sites and ontogeny of expression) suggests that the IGFBPs modulate IGF actions at specific target sites. Furthermore, because there is little change in the expression of IGFs or IGF receptor mRNA during fetal lung development, regulation of IGFBP expression may be essential to the control of IGF actions during lung development (76). Correlation of lung growth and maturation and the local production of IGFs have been poorly explored in humans. IGF mRNAs are expressed throughout gestation in the human fetal respiratory tract with a clear predominance of IGF II and a decreasing expression of both IGFs after the 20th week of gestation. IGFs are mainly detected in the mesodermal-derived components of the respiratory tract, especially in the undifferentiated mesenchyme of the lung beds up to 20 weeks of gestation (77).

In *in vitro* studies on isolated fetal pulmonary fibroblasts and type II alveolar epithelial cells, IGF-I slightly stimulated DNA accumulation in fibroblasts although it did not significantly stimulate thymidine incorporation, contrary to IGF-II which presented a dose-dependent stimulating activity of thymidine incorporation. Neither IGF-I nor IGF-II stimulated type II cell growth (78). IGFs thus appear to primarily control the growth of lung mesenchyme. IGFs play an important role in somatic growth in prenatal life. Mice carrying a null mutation of the IGF-I gene are dwarfed to 60% of normal birth weight (79). Depending on the genetic background, some of the IGF-I null mice die in the neonatal period, while others survive to reach adulthood. On the other hand, null mutants for the IGF-I receptor (IGF-IR) gene always die at birth of respiratory failure and exhibit a more severe growth deficiency. Dwarfism is also more exacerbated in IFG-I and IGF-II double null mutants and in IGF-IR and IGF-IIR double null mutants. Even though the lungs do not show a gross defect in primary branching morphogenesis per se, they appear hypoplastic. However, it also seems likely that IGF signalling may play a key role in facilitating signalling by other peptide growth factor pathways involved in lung morphogenesis, since IGF-IR signalling function is required for both the mitogenic and morphogenic activities of the EGF receptor (80).

Specific IGF binding proteins (IGFBP) modulate the action of these growth factors and their availability to their receptors. IGFBPs may be the mechanism for modulating the actions of the IGF peptides during the process of lung development. The generation of IGFBPs within developing organs, and their spatial arrangement, may determine IGF action at specific micro anatomic sites. The spatial distributions of IGFs are separately controlled, to some extent by endogenously generated IGF binding proteins (81). Six IGFBPs have been identified so far, designated IGFBP-1 through to IGFBP-6 (82). IGFBPs modulate IGF activity in several different ways. In circulation they act like carrier proteins and serve as a reservoir for IGF and in extracellular tissues, they control the transport and biologic activity of IGF on responsive target cells. IGFBPs produced by lung mesenchymal cells, fibroblasts and smooth muscle cells may be important in the regulation of IGF action for a number of different pulmonary cells. It has been demonstrated by immunohistochemical localization that IGFBPs result in the accumulation of IGF on pulmonary epithelium (83).

4.4. PDGF peptide and their receptors

The PDGFs are dimers of A- and/or B-chains that bind to and activate two related receptor tyrosine kinases, PDGFR [α] and [β]. PDGF-AA and PDGF-BB homodimers and PDGFRs are present in embryonic mouse lung and are differentially regulated in fetal rat lung epithelial cells and fibroblasts (84). PDGF-AA and PDGF-BB were localized to airway epithelial cells as early as 12 days gestation, 2 days before their appearance in mesenchymal cells, in fetal rat lungs (85). Immunoreactivity for both PDGF homodimers increased until the late pseudoglandular stage of lung development followed by fluctuations in reactivity during the canalicular stage and weak immunoreactivity to either PDGF homodimer during the saccular stage. The presence of

PDGF in both developing airway epithelial cells and mesenchymal cells, as well as gestation-dependent changes of PDGF homodimers, is compatible with a role for PDGF in fetal lung development (85). PDGF is also thought to play a major role in the stimulation of lung fibroblasts and hence modulate response to lung injury. Alveolar hypoxia increases gene expression of extracellular matrix proteins and PDGF-B in rat lung parenchyma. Variable expression of PDGF family proteins has been reported following bleomycin-induced acute lung injury (86). PDGF produced by alveolar macrophages modulates the expression of bFGF by lung fibroblasts (87). The ability of rat lung fibroblasts themselves to produce PDGF *in vitro* indicates that they may act by an autocrine mechanism in modulating the effects of fibroblasts during lung injury (87). Exogenous retinoic acid (RA) influences alveolarization by stimulating fibroblast proliferation through a PDGF-mediated autocrine mechanism, which is enhanced by RA and vitamin D when added in combination (88).

PDGF-AA regulates DNA synthesis and early branching in early mouse embryonic lung epithelium in culture (89). Abrogation of PDGF-AA expression with antisense oligodeoxynucleotide or PDGF-A blocking antibodies decreases DNA synthesis and hence the size of the early embryonic mouse lung in culture as well as interfering with early branch point formation (89). On the other hand, abrogation of PDGF-B chain expression with antisense oligodeoxynucleotide reduces the size of the epithelial component of early embryonic mouse lung explants, but does not reduce the number of branches. Knockout models of PDGF-B (90), PDGFR[HNF-3β] (91) have revealed important information concerning the normal physiological functions of the PDGFs during mouse embryonic development. The PDGF-B and –R[β] null phenotypes are embryonic lethal and strikingly similar (90, 91). At late gestation (E17-19) homozygous mutants develop generalized hemorrhage and edema; most of them die before delivery and the remaining die shortly after delivery. The underlying cause of bleeding is currently under investigation, however preliminary studies suggest a developmental defect in the blood vessel wall (92). Both PDGF-B and –R[β] knockout embryos display a developmental defect of the kidney glomerulus that consist of a complete lack of mesangial cells. About 50% of PDGF-A homozygous null mutant mice die prenatally before E10; those surviving beyond E10 develop increasing growth retardation and are less than 50% the size of heterozygote or wild type littermates by two weeks of age. Surviving PDGF-A -/- mice develop pulmonary emphysema secondary to a failure of alveolar septation (93). This phenotype is apparently caused by loss of alveolar myofibroblasts and associated reduction in elastin fiber deposition. Since PDGF-a receptors are expressed in the lung at the location of putative alveolar myofibroblasts, and the latter were especially absent in PDGF-A null mutants, it appears that PDGF-A chain expression is essential for the ontogeny of pulmonary alveolar myofibroblasts. Thus PDGF signalling appears to play a permissive role for epithelial DNA synthesis during embryonic life and an instructive role for the ontogeny of myofibroblasts, elastin synthesis by the latter cell lineage and hence alveolarization in postnatal life. The analogy between the mesangial cell deficiency in PDGF-B knockouts and the alveolar smooth muscle cell (SMC) deficiency in PDGF-A knockouts is striking and may suggest a common role for PDGFs in the development of certain type of connective tissue cells. Absence of mesangial cells and alveolar SMCs leads to failure of involution of the epithelial sheet, consisting of podocytes in the kidney and pneumocytes in the lung. The involution of the epithelial sheets is a direct consequence of extracellular matrix production (mesangial matrix and elastin respectively) by mesangial cells and alveolar SMCs, which creates a large surface area for filtration and gaseous exchange respectively. Localized elastin deposition in the wall of the prealveolar saccules is likely providing the mechanical driving force (elastic sphincters) leading to compartmentalization of the prealveolar saccules into alveoli. This may contribute to the pathogenesis of emphysema. The imbalance between elastases and elastase inhibitors as in α_1 antitrypsin deficiency or as a result of increased local concentration of elastases as in the lungs of smokers in chronic bronchitis, lead to the same end result, emphysema, secondary to breakdown of septal elastin. Thus breakdown of septal elastin (as in α_1 antitrypsin deficiency or chronic bronchitis) and failure of septal elastin deposition during development (as in PDGF-A mutants), may explain the pathogenesis of emphysema (92).

a_2-macroglobulin (a_2M), a binding protein and a protease inhibitor regulates the biologic activity of several growth factors and cytokines (94). Plasma a_2M accounts for 3% to 5% of total proteins and is primarily synthesized in the liver (95), while small amounts of this protein are synthesized by microglia as well (96). They act as modulators of acute phase response (97) and bind to and moderate the function of cytokines (97, 98), a variety of growth factors (99) and potent proteases (100). Cytokines that bind to a_2M include PDGF, TGFß1 and 2, bFGF, EGF and VEGF and interleukins (IL-1B, IL-6, IL- TGFß8). PDGF binds both native and proteinase- or amine-activated forms of a_2M (22), and PDGF-stimulated fibroblast proliferation (22) and chemotaxis are inhibited by native a_2M (101). Native a_2M (which is not receptor recognized) binds to and prevents PDGF from binding to the receptor (102). This conformational form of a_2M serves as an extracellular reservoir for the growth factor, where it can be released over time in a controlled fashion to interact with the PDGF-a or –ß receptor (103). Two receptor-recognized forms of a_2M differentially modulate PDGF. a_2M activated with methylamine synergistically enhances the growth promoting activity of PDGF purified from human platelets in the nanomolar range (22). Conversely, a_2M activated with proteases inhibits PDGF-BB-induced fibroblast proliferation (104).

SPARC (secreted protein, acidic and rich in cysteine) is an extracellular matrix protein, that is prevalent in areas of active tissue morphogenesis and remodeling, suggesting that it possesses a function related to cell proliferation, migration and differentiation (105). SPARC

was found to bind and regulate the activity of PDGF-B chain molecules (-BB and –AB) but not PDGF-AA molecule (106). The potential importance of SPARC in the lung is unknown. Because activated lung macrophages express abundant PDGF-BB (107), it is possible that SPARC could be an important modulator of fibroblast and smooth muscle cell proliferation after airway injury.

4.5. TGFb **peptides and their receptors**

Members of the TGFβ family exert a wide range of biological effects on a large variety of cell types regulating cell growth, differentiation, matrix production and apoptosis. Many of them have important functions during embryonal development in pattern formation and tissue specification and in the adult they are involved in processes such as tissue repair. They initiate their cellular action by binding to receptors with intrinsic serine/threonine kinase activity. This receptor family consists of two subfamilies, type I and type II receptors, which are structurally similar, both of which are needed for signalling by TGFβ. TGFβ1, TGFβ2 and TGFβ3 peptides and the TGFβ type I and type II receptors are expressed and differentially distributed in the embryonic and fetal lung (108, 109). TGFβ1 and TGFβ2 both inhibit pulmonary branching morphogenesis in culture, although TGFβ2 is considerably more potent than TGFβ1 (44, 109). The expression of pRb is not necessary for the inhibitory effects of TGFβ1 on branching to be transduced, since TGFβ1 inhibits branching in pRb -/- embryonic lungs to the same extent as in wild type lungs. However, TGFβ1 suppresses n-myc expression in wild type lungs but not in the pRb null mutant, indicating that TGFβ is necessary for the inhibitory effect on n-myc expression to occur (110). Interestingly, gene targeting of the n-myc locus has produced a hypoplastic, neonatal lethal lung phenotype as well as lethal hypoplasia of subendocardial fibroblasts (111). Perhaps the n-myc knockout prevents the pulmonary epithelium from expanding to a sufficient surface area to support gas diffusion postnatally. TGFβ3 null mutation also results in a specific immature-appearing neonatal lung phenotype, which is rapidly fatal in newborn mice (112). Unlike the normal neonatal lung phenotype found in TGFβ1 null mutant mice, which has been attributed to maternal transplacental rescue, the TGFβ3 null mutation appears to be refractory to maternal transplacental rescue. TGFβ3 gene expression is also strongly induced in response to corticosteroid treatment of fetal lung fibroblasts (113), suggesting the hypothesis that the well recognized maturational effects of glucocorticoid on late fetal lung may in part be mediated by stimulation of temporospatially restricted TGFß3 gene expression.

TGFβ2, but not β1 or β3, regulates pattern formation during early rat lung organogenesis (114). Inhibition of TGFβ2, but not TGFβ1 and TGFβ3, with antisense oligonucleotides and neutralizing antibodies resulted in significant inhibition of early lung branching in culture. Addition of minute amounts of exogenous TGFβ2 but not β1 or β3 restored the branching of TGFβ2 antisense-treated explants. Higher concentrations of TGFβ2 were inhibitory, but lower concentrations of TGFβ2 increased thymidine uptake by isolated epithelial cells (114). Combination of TGFβ1 and EGF resulted in a more normal branching pattern of fetal mouse lung organ cultures than either of them alone, suggesting a mutually interactive mechanism that regulates lung branching (35). Upregulation of TGFβ receptors (TGFβRI and TGFβRII) in hyperoxia-induced lung injury in rat lungs, suggests that TGFβ receptors may regulate interactions between epithelium and mesenchyme (20) in lung injury. TGFβ1 increases elastin gene expression in cultured neonatal rat lung fibroblasts (115). Interference with the function of endogenous TGFβ1 in cultured lung fibroblasts, through the addition of neutralizing antibodies or antisense oligonucleotides, decreases tropoelastin and tropoelastin mRNA production in these cells, suggesting its role in lung repair. Evidence from normal and targeted misexpression studies in mice suggests that BMP-4, another TGFß family peptide, also plays a role in embryonic lung morphogenesis (116). Misexpression of BMP-4 driven by the SP-C promoter in transgenic mice results in lungs that are smaller than normal with grossly distended terminal buds and large air filled sacs at birth. Targeted misexpression of TGFβ1 using the same SP-C promoter system also results in a neonatal lethal, hypoplastic pulmonary phenotype with decreased saccule formation and epithelial differentiation (117). Taken together, these findings suggest that TGFβ family peptide overexpression *in vivo* merely reflects a default negative regulatory effect on morphogenesis similar to that elicited in culture (44, 109, 110). On the other hand, abrogation of TGFβ type II receptor signalling with antisense oligodeoxynucleotide or with blocking antibodies stimulates lung morphogenesis two to three fold and increases the expression of TTF-1 and SP-C (109). Thus endogenous autocrine-paracrine TGFβ signalling through the TGFβ type II receptor appears to negatively regulate lung organogenesis. The negative effect of TGFβ signalling through the TGFβ type II receptor on cell cycle progression in pulmonary epithelial cells probably plays a major role in the latter inhibitory effect by limiting expansion of the epithelial surface area (3).

TGFβ signalling through the TGFβ type I receptor specifically instructs the formation of branch points (3). Abrogation of the TGFβ type I receptor with antisense oligodeoxynucleotide significantly reduces the formation of branch points by E11 early embryonic mouse lung in culture, resulting in a phenotype characterized by long, tubular appearing airways devoid of new branch points. This effect is associated with decreased fibronectin and matrix Gla protein gene expression and the failure to form condensations of extracellular matrix containing fibronectin as would be expected to occur at sites where a new branch point is about to form (118). The growing points of tubular appearing airways are virtually devoid of fibronectin. Thus, the regulation of fibronectin gene expression by the TGFβ type I receptor apparently plays a key role in the molecular basis of lung morphogenesis by instructing the formation of new airway branch points and the localized deposition of extracellular matrix components (3). TGFß ligand-interacting proteins and proteoglycans such as betaglygan (TGFβ type III receptor), biglycan,

decorin, fibromodulin and endoglyn may affect pulmonary morphogenesis (119). Endoglyn is a dimeric TGFβ1 and TGFβ3 binding protein of endothelial cells which modulates cellular responses to TGFβ1 and can form heteromeric complexes with TGFβ signalling receptors (120). Interestingly, endoglyn is the gene for Osler-Weber-Rendu hereditary telangiectasia type I, a condition characterized by large intrapulmonary malformations (121). It is also interesting to note that massively dilated pulmonary vessels with thin or absent smooth muscle layers are a prominent pathologic feature of the neonatal lethal TGFβ3 null mutant phenotype (112). Abrogation of betaglycan expression using antisense oligodeoxynucleotide gene knockdown stimulates lung morphogenesis in culture and strongly inhibits the effectiveness of exogenous ligands, particularly TGFβ2, to inhibit lung morphogenesis in culture (122).

The recent identification of the Smad family of signal transducer proteins has unravelled the mechanisms by which TGFβ signals from cell membrane to the nucleus. Briefly, following ligand binding to the TGFβ type II receptor, the TGFβ I receptor is recruited to enter a heteromeric complex with TGFβ/TGFβIIR, placing the TGFβIR in position to be trans-phosphorylated by the constitutively active TGFβIIR intracellular serine/threonine kinase domain. Smads 2 and 3 can bind the TGFβ receptor heteromeric complex and are phospharylated by the activated type I receptor serine/threonine kinases. Activated Smads 2 and 3 then form complexes separate from the receptor with smad 4, which as a result of association with activated Smads 2and 3, can translocate to the nucleus, where it acts as a transcriptional activation or repression factor at specific promoters (123). Smads 6 and 7 are inhibitors of activation of Smads 2 and 3. Smad 7 expression is rapidly induced following TGFβ ligand signalling and is considered to function as a negative feedback element in the TGFβ signalling pathway. Abrogation of Smads 2 and 3 or 4 expression results in a strong gain of function phenotype for lung branching morphogenesis of early murine embryonic lung in culture, similar to that obtained after abrogation of either TGFβIIR or IIR signalling (122, 112). Thus pathway restricted smads (2, 3, 5) and inhibitory smads (11, 12) play a crucial role in embryonic development in regulating TGFβ signalling.

Inhibition of lung epithelial cell growth by TGFß2 is blocked by a_2M, but a_2M does not block the mitoinhibitory effect of TGFß1 (124). Even though the function of a_2M in regulating TGFß action is not well understood, in plasma it serves as a clearance mechanism for TGFß1 (125). It appears to serve as a negative modulator of TGFß induced biological effects on epithelial cells. Native a_2M, which does not bind the a_2M receptor/LRP, does not enhance TGFß1 stimulated smooth muscle cell mitogenesis, and TGFß bound to this form of a_2M could serve as an extracellular reservoir for the growth factor (126). However, fast or receptor recognized a_2M that has been modified with methylamine synergistically enhances TGFß1-induced mitogenesis of vascular smooth muscle cells. (127). This high molecular weight complex of TGFß and binding protein should not be confused with the high molecular weight latent TGFß complex. The lung cells secrete TGFß and the bioactive (25-kDa) TGFß is cleaved from the latent complex by proteinases or low pH (128). The 25-kDa active TGFß interacts with at least three different cell surface receptor-like molecules (type I, II and III) that possess signalling properties for diverse biological actions of TGF- ß (129), or the growth factor can bind a_2M in the extracellular environment. Because proteinases trigger the slow to fast conformational change in a_2M, they could mediate the clearance of TGFß through the a_2M-receptor/LRP. If proteinase activity is low, the TGFß1/a_2M complex would not be converted to its receptor-LRP recognized form. In this case, TGFß could be released by factors such as heparin or reduced pH and targeted to TGFß cell surface receptors (130).

Extracellular matrix (ECM) components bind and serve as a storage depot for several growth factors, including betaglycan and decorin for TGFß (131) and heparin and glycosaminoglycans for FGF (132). The diverse group of ECM molecules includes adhesive proteins (collagen, laminin and fibronectin) and antiadhesive proteins such as tenascin, thrombospondin and secreted protein, acidic and rich in cysteine (SPARC). Although structurally dissimilar, these glycoproteins modulate cell shape. Some of these glycoproteins also bind and sequester growth factors. Growth factors bound in this manner might interact directly with cells or remain in the extracellular matrix before release by enzymes such as heparinase and plasmin to function as soluble growth regulators. TGFß Type III receptor is a membrane proteoglycan, also exists in soluble form and thus acts as cytokine binding protein (129). Decorin and betaglycan are proteoglycans present in the extracellular matrix that serve as binding proteins for TGFß (131). It is possible that TGFß/decorin complexes may be internalized by the cell surface receptor that recognizes decorin, as has been shown for TGFß1/a_2M complexes internalized through the a_2M receptor. It has been suggested that neutralization of TGFß activity with decorin might allow for therapeutic intervention in cases where excessive TGFß activity causes a fibrotic reaction (131).

4.6. vascular endothelial growth factor (VEGF)

Vascular Endothelial Growth Factor (VEGF) is a potent endothelial cell mitogen (133) involved in both normal and abnormal angiogenesis (134). VEGF is expressed in lung epithelial cells and its expression is regulated by both oxygen and cAMP in the developing human lung (134). VEGF mRNA and protein is localized to the distal airway epithelial cells and VEGF protein to the basement membrane subjacent to airway epithelial cells (134). Localization of VEGF to the basement membrane of airway epithelial cells may be important for directing capillary development in the human lung. VEGF and its specific receptors, fetal liver kinase receptor (flk-1), and fms-like tyrosine kinase receptor (flt-1) are upregulated during the development of capillaries in fetal and newborn rat lung (135). Endothelial cells, which were smooth muscle actin negative, developed into a capillary network

surrounding the budding components of distal airways during the mid-pseudoglandular phase before communicating with proximal vessels in developing human lung (136). Endothelial cells of the capillary network are mainly positive for vWF (von Willebrand Factor) during the early gestational stages, as opposed to the phenotype of those of mature lungs (vWF negative and thrombomodulin positive) (136). VEGF immunoreactivity was detected in the cytoplasm of airway epithelial cells throughout the branching bronchi and was observed in the budding component of airways in the canalicular phase lung. Endothelial cells of the capillary network surrounding the distal airway developmentally altered their characteristics to be thrombomodulin (TM) predominant phenotypes. Capillary endothelial cells around the budding components of distal airways may therefore be exposed to relatively high concentrations of VEGF, resulting in the acquisition of TM-predominant phenotypes in these endothelial cells (136). Expression of TM and TM-dependent anticoagulant activity is regulated by VEGF (137). These findings suggest that diffusely distributed VEGF in pulmonary epithelial cells is involved in endothelial proliferation and the maintenance of vascular structure and relatively abundant VEGF in the budding components of distal airway may change the endothelial cell phenotype to a mature alveolar capillary vessel type. This process of epithelial-endothelial interaction may be important in establishing the sufficiently wide and functional blood-gas interface.

VEGF may be an important autocrine growth factor for distal airway epithelial cells in the developing human lung (138). VEGF mRNA and protein are abundant in distal epithelium of mid-trimester human fetal lung. Addition of VEGF to human fetal lung explants resulted in increased epithelium volume density and lumen volume density. Cellular proliferation was prominent in distal airway epithelial cells and increased in VEGF treated explants. VEGF treated explants also demonstrated increased SP-A mRNA, SP-C mRNA and SP-A protein levels compared to controls. VEGF, largely produced by type II cells, can induce vascular leak resulting in pulmonary edema. VEGF produced by distal airway epithelial cells and its localization to the basement membrane subjacent to the epithelium in explant culture (134) may be important in the development of the pulmonary capillary bed resulting in the formation of functional blood-gas interface.

Human neonatal deep pulmonary lavage VEGF tripled between day 1 and 3 of age; at day 7 of age VEGF levels were higher in dexamethasone treated subjects than in controls in a randomized, controlled dexamethasone trial (139). Higher lavage VEGF levels on days 1 and 3 were also correlated with lower gestational age at birth. VEGF levels in BAL/tracheal aspirates are low at birth and increase with increasing postnatal age in preterm infants (27, 140). Disordered and decreased expression of VEGF, in the presence of alveolar damage, may contribute to abnormal pulmonary vasculature in infants dying with BPD (141). Dexamethasone has been shown to block the 3-fold increase in VEGF mRNA that occurs in cultured pulmonary artery smooth muscle cells exposed to hypoxia (142). VEGF protein abundance is significantly greater in term newborn lung than it was in fetal lung, whereas there is no appreciable difference in VEGF expression in the lungs of lambs with BPD compared to fetal lungs (143). It is possible that dexamthasone and age-associated changes in VEGF may affect pulmonary angiogenesis.

Hyperoxia exposure of 100% oxygen in neonatal rabbits dramatically decreases lung VEGF mRNA, alveolar epithelial cell VEGF expression, and VEGF immunostaining, with rebound during recovery (144). This depression of VEGF may contribute to impaired postnatal microvascular development in oxygen injury. Hyperoxia also decreases the expression of VEGF and its receptors in adult rat lungs (145), which may contribute to the pathophysiology of oxygen-induced lung damage. In rabbits, the proportion of the 189-amino acid VEGF mRNA, which codes for an isoform that binds to the extracellular matrix, increases fivefold during development and during neonatal oxygen injury, its expression declines and then returns to control values during recovery (146). VEGF protein is barely detectable at the 50% lethal dose time point then increases 10-40 fold in newborn or adult recovering animals. The signficiant injury-induced changes that occur in VEGF, particularly in the epithelial compartment, are likely to have long-term effects on the pulmonary vasculature as the neonatal lung is developing postnatally.

4.7. Additional factors

Gastrin-related peptide (GRP) is the mammalian equivalent of Bombesin and is produced by pulmonary neuroendocrine cells in adult lungs. Expression of GRP peaks in the rapidly proliferative phase of airway epithelial development. GRP receptors are expressed on airway epithelial cells in developing fetal lung and the interaction of GRP with GRP receptors stimulates airway development (147). At earlier stages of development in the mouse, GRP is expressed in undifferentiated epithelial lineage precursor cells (148). Blockade of GRP action by immunoperturbation or pharmacological blockers both *in vivo* and in culture retards lung epithelial development (149). Bombesin stimulates branching morphogenesis and lung maturation in culture and *in vivo* (150). GRP increases surfactant and non-surfactant phosphatidylcholine (PC) biosynthesis by type II cells at concentrations below 10^{-9} M although it did not affect DNA synthesis (78). Pulmonary nueroendocrine cell (PNEC) clusters in newborn hamster lung are focal points for regions of increased cell division, supporting the idea that pulmonary neuroendocrine cells produce one or more paracrine growth factors that stimulate proliferation of the surrounding lung epithelium (151).

Hepatocyte growth factor (HGF), a ligand for Met tyrosine kinase, is a mesenchyme-derived factor, which has mitogenic, motogenic and morphogenic activities on various epithelial cell types and is considered to be a possible mediator of epithelial-mesenchymal interaction during organogenesis and organ regeneration. C-met/HGF receptor mRNA was localized in lung

epithelial cells, whereas HGF mRNA was localized in lung mesenchymal cells in rat fetal lungs (152, 153). In organ cultures, exogenously added HGF apparently stimulated branching morphogenesis of the fetal lung. In contrast, HGF translation arrest or neutralization assays using antisense oligonucleotides resulted in clear inhibition of epithelial branching in cultured rat lung embryos, whereas recombinant HGF stimulated branching (153). In mesenchyme free cultures, HGF alone did not induce epithelial morphogenesis; however addition of both HGF and acidic FGF (aFGF, FGF1) or KGF (FGF7), ligands for the KGF receptor, induced epithelial branching more extensively than that was observed in explants treated with aFGF or KGF alone. In addition, the simultaneous inhibition of HGF and FGF mediated signalling using neutralizing antibody and antisense oligo-DNA resulted in drastic impairment of epithelial growth and branching (152). Thus, HGF seems to be at least one of the mesenchyme derived factors that supports branching morphogenesis in lung development.

Little is known about how retinoic acid (RA) metabolism is regulated in embryonic lung. It has been shown that RA signalling is essential for early lung bud formation and branching morphogenesis, but not for distal branching (154). RA treatment of rats from day 3 to 14 of age during hyperoxia exposure significantly attenuated the hyperoxia-induced inhibition of lung septation (155). Vitamin A stores are high in fetal lung and decrease toward term, possibly being utilized for changes in lung morphogenic remodeling (156). Retinoic acid may act as a co-factor for other growth factors in regulating normal lung morphogenesis.

The extracellular matrix (ECM) plays an important role in strengthening the alveolar capillary interface, which is attributable to the type IV collagen in the basement membrane (BM). When the capillary wall stress is high (either due to pulmonary hypertension or lung inflation), ultrastructural changes occur in the capillary wall, a condition known as stress failure. Recent experimental work suggests that rapid changes in gene expression for extracellular protein and growth factors occur in response to an increase in capillary wall stress. Alveolar hypoxia increases gene expression of extracellular matrix proteins and PDGF-B in rat lungs (38). High lung inflation for 4 hours increases mRNA levels of extracellular matrix (ECM) components (type III & type IV procollagen, fibronectin) and certain growth factors (FGF2, TGFβ1) in lung parenchyma in rabbit lungs (38). ECM components not only support tissue architecture, but also play a direct role in the modulation of cell proliferation and cell differentiation (3). Absence or inhibition of the interaction of epithelial cells with the basement membrane has a direct consequence in the failure of normal lung development (157). Adult alveolar basement membrane contains functional and structural domains that determine sites at which type I cells and type II cells localize (158).

5. SUMMARY

Even though it is important to study the effects of individual growth factors on isolated cell preparations, there are multiple growth factors present *in vivo* at different times during lung development and during the lung injury and repair process. The complex interactions among growth factors and the spatial and temporal relationship to cellular proliferation and differentiation might be different *in vivo* and is as yet unclear. The effects of a particular growth factor may be different at different sites and at different time points. The dynamic interplay among type II cells, the extracellular matrix and various growth factors may determine multicellular functions and play an important role in normal lung development and in repair of the lung epithelium following injury. A better understanding of the epithelial-endothelial interaction and regulation of the alveolar-capillary interface will provide important clues for novel therapies in preterm infants with chronic lung injury. Transgenic mouse models generated by gene targeting and gene addition have been very useful for the analysis for gene function *in vivo* and are providing unexpected insights into pathways mediating lung morphogenesis and repair. HNF-3β and TTF-1 are involved in gene regulation and formation of the respiratory epithelium. Precise regulation of TTF-1 is critical for homeostasis in the postnatal lung. Abnormal expression of TTF-1 in various congenital disorders of the lungs is beginning to be understood. Newer therapeutic targets are being identified from candidate gene studies based on the molecular embryology of the lung. These targets are quite likely to be amenable to rational therapeutic intervention to prevent or ameliorate the effects of abnormal signalling that culminate in human lung disease. Cytokines bound to a particular protein are protected from degradation and it is reasonable to speculate that dysfunction of the clearance mechanism may lead to overstimulation of alveolar macrophages and this could be the key in understanding inflammatory lung diseases that involve cytokine overexpression. Epithelial-mesenchymal interactions and their regulation by growth factors and various cytokines, both during normal development and in disease, in an *in vivo* setting, needs to be better understood, which may pave the way for better treatment of acute lung injury and chronic lung disease in the future.

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Abbreviations: aFGF: Acidic fibroblast growth factor, α2M: Alpha-2 Macroglobulin, bFGF: Basic fibroblast growth factor, BMP-4: Bone morphogenetic protein 4, BPD: Bronchopulmonary dysplasia, CLD: Chronic lung disease, EGF: Epidermal growth factor, EGFR: Epidermal growth factor receptor, FGF: Fibroblast growth factor, FGFR: Fibroblast growth factor receptor, GRP: Gastrin-related peptide, HGF: Hepatocyte growth factor, HNF-3b: Hepatocyte nuclear factor 3b, IGF: Insulin growth factor, IGFBP: Insulin growth factor binding protein, IGFR: Insulin growth factor receptor, IL: Interleukin, KGF: Keratinocyte growth factor, KGFR: Keratinocyte growth factor receptor, PDGF: Platelet derived growth factor, PDGFR: Platelet derived growth factor receptor, RDS: Respiratory distress syndrome, SP: Surfactant protein, TGFα: Transforming growth factor alpha, TGFβ: Transforming growth factor beta, TTF-1: Thyroid transcription factor –1, VEGF: Vascular endothelial growth factor

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