Coordination of Genetic, Epigenetic, and Environmental Factors in Lung Development, Injury, and Repair*

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Lung development is regulated by a number of genetic and epigenetic factors including transcriptional factors, peptide growth factor receptor-mediated signaling, and environmental influences including lung fluid volume, mechanical strain, and hypoxia. These inputs are integrated during the normal process of lung organogenesis to determine organized patterns of cellular proliferation and cell lineage differentiation and to correlate structure with physiologic function. Lung development extends from branching morphogenesis in early embryonic life, through the critical transition from fetal life to air breathing, up to the completion of alveolarization which occurs postnatally. Lung organogenesis can be positively or negatively impacted by environmental factors.

Transcription of master genes is necessary in the process of pulmonary organogenesis. The pattern-forming events that control the initial and subsequent steps of pulmonary organogenesis are only now beginning to emerge. We and others have recently demonstrated that the thyroid transcription factor-1 (TTF-1) family, also called the thyroid enhancer binding protein (Tebp) transcriptional factor family, is essential for the induction of embryonic lung branching morphogenesis.1,2 TTF-1 con-
sensus recognition sites are found in the 5′ promoters of several peripheral lung cell lineage specific genes, including surfactant protein-A (SP-A), SP-B, SP-C, SP-D, CC-10, and TTF-1 itself.3-8 However, while TTF-1 expression appears to be necessary for lung morphogenesis, other families of transcription factors are also clearly involved.

The hepatocyte nuclear factor (HNF) family of transcription factors are related to the forkhead family of Drosophila and are known to play key roles in regional specification of epithelial cell fates in the gastrointestinal tract and liver. HNF-α is expressed in the gut epithelium anterior to the liver and consensus HNF binding sites are found in the 5′ promoter regions of peripheral lung-specific genes including SP-A, SP-B, SP-C, SP-D, and CC-10 in close proximity to TTF-1 sites.49 Thus, the HNF family appears to cooperate with the TTF-1 family to determine pulmonary epithelial cell lineage fates.10,11

Very recently, tracheal less (trh) has been identified as necessary to direct tubulogenesis in the respiratory organs and salivary glands of Drosophila.12 In trh mutants, tube-forming cells of the trachea, salivary gland, and filzkörper fail to invaginate to form tubes and remain on the embryo surface.13 The trh expression is controlled by Sex combs reduced (scl) and forkhead (fkh), and is homologous to the human hypoxia-inducible factor-1α.14 It will be interesting to determine whether mammalian homologues of these additional Drosophila gene families also play a role in pulmonary organogenesis.

Peptide growth factor signaling is also necessary for lung morphogenesis. Branching morphogenesis and cell lineage differentiation also occur spontaneously in mouse early embryonic lung under serumless chemically defined conditions.15-17 Soluble factors released by peripheral lung mesenchyme can induce ectopic branching from the trachea of early mouse embryonic lung explants, as well as inducing expression of a complete repertoire of genes specific to peripheral lung epithelium including SP-A, SP-B, SP-C, and CC-10.18 These data strongly suggest that endogenous factors can activate both morphogenesis and lung-specific gene expression. Candidate inductive peptide growth factors include epidermal (EGF), insulin-like (IGF), basic fibroblast (bFGF), platelet-derived (PDGF), hepatocyte (HGF), keratinocyte (KGF), and transforming (TGF)-β3, all of which exert inductive or permissive influences on lung development as demonstrated by gain and loss of function experiments in early embryonic mouse lung organ culture, transgenic mice, and null mutant mice.19-26

In general, peptide growth factor cognate receptors with tyrosine kinase intracellular signaling domains such as EGF stimulate lung morphogenesis, while those cognate receptors with serine/threonine kinase intracellular signaling domains, such as the TGF-β family are inhibitory.20,22,25,27-31 For example, we have recently found that branching morphogenesis is reduced by 50% both in vivo and in vitro in the lungs of epidermal growth factor receptor (EGFR) null mutant mice.23 While addition of exogenous EGF stimulates branching threefold and SP-C levels 50-fold in wild-type embryonic lungs in culture, whereas the EGFR null mutants do not respond at all to exogenous EGF. In contrast, exogenous TGF-β1 or TGF-β2 suppresses branch-

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ing morphogenesis, overrides the stimulatory effects of exogenous EGF, and decreases TTF-1 and SP-C expression. In addition, abrogation of TGF-β type II receptor signaling, either with antisense oligodeoxynucleotides or with blocking antibodies, stimulates lung morphogenesis twofold to threefold and increases expression of TTF-1 and SP-C. Thus, excess TGF-β signaling appears to default in negative regulation of lung organogenesis. However, TGF-β3 null mutation results in an immature appearing neonatal lung phenotype which is rapidly fatal in newborn mice.8 Unlike the normal neonatal lung phenotype found in TGF-β1 null mutant mice, which has been attributed to maternal transplacental rescue, TGF-β3 null mutation appears to be refractory to maternal transplacental rescue. TGF-β3 gene expression is also strongly induced in response to corticosteroid treatment of fetal lung fibroblasts,32 suggesting the hypothesis that the well-recognized maturation enhancing effects of glucocorticoids on late fetal lung may in part be mediated by stimulation of temporospatially restricted TGF-β3 gene expression.

Organized cellular proliferation can also be both positively and negatively regulated by environmental factors including lung liquid volume/mechanical strain and hypoxia. During recovery from hypoxia and in some forms of experimental lung hypoplasia which are due to reduced lung volume, impaired morphogenesis and lung size may be associated with alveolar type II epithelial cells (AECII) proliferation. The signaling pathways involved are only just beginning to be unraveled, but it is already clear that autocrine and paracrine peptide growth factors are of crucial importance in the responses to lung injury. Ion channels may be important in transducing morphogenetic stimuli, particularly in relation to the regulatory role of lung liquid volume/mechanical strain.

Distending pressure has long been known to play a key role in determining lung growth, yet the molecular mechanisms connecting fluid pressure to cellular proliferation are largely unknown. Increasing the distending pressure by increasing outflow resistance can partially reverse lung hypoplasia in experimental diaphragmatic hernia,32 or oligohydramnios,34 and causes hyperplasia when the lung is initially normal.35 These data suggest that lung liquid volume regulation is crucial for "quantitative" lung growth,36 and that distension is linked to cell growth. Little is known about this except that TGF-II mRNA level varies inversely with lung volume.37-38 Recently, calcium entry through mechanical strain-activated ion channels has been shown to play a critical role in fetal lung epithelial cell proliferation.39 Activation of phospholipases and protein kinase C by PDGF-B and its receptor have also been implicated in the initiation of downstream strain-initiated events.40 EGF has also recently been shown to regulate both alveolar epithelial junctional permeability and active sodium transport as well as Na⁺/K⁺-ATPase α1 and β1 subunit expression.41 Interestingly, the α-amiloride-sensitive epithelial sodium channel /−/− mouse dies in the neonatal period from failure to clear lung liquid,42 further confirming that factors that regulate lung liquid are essential both for normal lung development and the smooth transition to air breathing.

Hyperoxia, even the "natural hyperoxia" attending human premature delivery, has long been considered a major etiologic factor in the adverse impact of premature human delivery on lung development. Following acute hyperoxia in adult rodent models, the alveolar epithelium is denuded. Alveolar type II cells then regain the capacity to proliferate and repopulate the gas diffusion surface.43 Thus, in this special situation, AECII may be considered to have regained a stem cell function for alveolar epithelial repair.44 On the other hand, in neonatal rodents, acute hyperoxia results not only in proliferation of AECII to repair the denuded epithelium, but also in a significant inhibition of alveolar development.45 Alveolar hypoplasia is also an important sequela of neonatal hyperoxia both in primates and in human premature infants.46

Candidate molecular switches in the transition from a quiescent to a proliferative alveolar epithelial cell phenotype and back again following acute hyperoxia include autocrine peptide growth factor signaling pathways and cell cycle regulatory elements. TGF-β3 is the major TGF-β peptide secreted by rat AECII in culture, and it exerts an autocrine-negative regulation on adult AECII proliferation. The production of TGF-β3 by these cells is dynamically downregulated during the proliferative phase of recovery from acute hyperoxia but returns to normal levels following completion of recovery. Thus, the rate of DNA synthesis in rat AECII is inversely proportional to the autocrine production of TGF-β3.48

In the neonatal rat, hyperoxia results in a profound inhibition of alveolarization and is associated with high levels of TGF-β (mostly TGF-β1) activity in bronchial lavage. We have confirmed recently that human premature babies’ lavage samples are also unusually rich in TGF-β activity, which leads us to postulate that excess TGF-β1 production may play a key role in adversely regulating the normal temporospatial pattern of lung-specific gene expression.

We have also recently discovered that cyclin D2 and Cdc2 arrest specifically downregulated in quiescent adult AECII.49 During the proliferative recovery phase following acute hyperoxia, these genes are reinduced, with activation of cdk4.45 These critical cell cycle control genes are also downregulated by TGF-β1 and cell-cell contact,50 further supporting a key role for TGF-β signaling in modulating the proliferative response to acute hyperoxia and the restoration of quiescence following recovery.

There are novel rational pharmacologic and gene therapeutic approaches. Organ regeneration has long been a biological reality in human skin wound healing and holds promise in human liver. In rodents, lung regeneration has long been known to occur following lobectomy. However, whether lung regeneration can occur in primates or humans is controversial. Based on clinical experience with follow-up of human premature infants with bronchopulmonary dysplasia, extensive lung growth and lung regeneration can occur naturally in these children. The issue of whether adult lung tissue, such as a parental lobe transplanted into a young person, can grow or regenerate remains to be settled but would be therapeutically desirable. Also, in cases of severe congenital pulmonary hypoplasia or dysplasia, for example, diaphragmatic hernia and cystic adenomatoid malformation, lung regeneration could...
make the difference between nonsurvival and eventual recovery in up to 40% of cases.

In preliminary attempts to achieve our rational therapeutic goals, we have modeled the molecular structure of the TGF-β2 dimeric ligand interaction with the TGF-β I and II receptor ternary complex. This theoretic structure provides a rational framework for developing antibodies and cyclic peptides which can perturb TGF-β ligand-receptor interaction. Based on these structural biology computer modeling studies, we have devised an N-terminal TGF-β type II receptor peptide polyclonal antibody which perturbs specific features of TGF-β ligand-receptor interaction. This antibody abrogates TGF-β1 interaction with TGF-β type I receptors and perturbs interaction of ligand with the TGF-β type II receptor.51 The perturbation prevents assembly of ternary TGF-β type I and type II ligand-receptor signaling complexes. Preincubation of fetal milk lung epithelial cells with this antibody completely abrogates activation of the plasmogen activator inhibitor-1 promoter by TGF-β1, but inhibition of DNA synthesis by TGF-β1 is not adversely affected. Thus, the antibody appears to distinguish between TGF-β type I receptor-mediated activation of extracellular matrix protein regulatory promoters and inhibition of the cell cycle through the TGF-β type II receptor. This antibody also has the interesting property of stimulating embryonic mouse lung morphogenesis in vitro.31

Our ultimate future aim is to devise new rational and gene therapeutic approaches to ameliorating lung injury and augmenting lung repair. The strategic approach will therefore be to develop rational therapeutic agents or gene therapies that will regulate gene products or genes shown to play key roles in these processes. The ideal agent or agents would therefore mimic the instructive role of lung mesenchyme and would correctly induce the temporal-spatial pattern of lung-specific gene expression necessary to instruct lung regeneration.

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Polycationic Lipid-Mediated Gene Transfer to the Abnormal Pulmonary Circulation*

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Novel treatments for acquired diseases may be accomplished by the transfer of therapeutic genes into somatic cells. This report demonstrates gene transfer to the lung circulation with a novel polycationic liposome from an imadazolium derivative with the formula CH₃(CH₂)₇CH=CH(CH₂)₂⁺ (lipid B).

Mice were injected intravenously with lipid B complexed with plasmids containing either chloramphenicol acetyltransferase (CAT) or lac-Z complementary DNA driven by the cytomegalovirus promoter/enhancer. Two days after lipid B transfection, normal lung tissue showed the greatest CAT activity with heart and spleen having, respectively, 10% and 6% of lung activity levels. Comparing lipid B to DOSPA/DOPE/Lipofoctamine, lung CAT activity was 4 orders of magnitude greater in the lipid B transfected lungs. Immunostaining of formalin-fixed lung sections for the lac-Z gene product β-galactosidase

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